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Studies on the Pathogenicity of Brucella Suis for Cattle. II

F. V. WASHKO, D.V.M., M.S., Ph.D., and L. M. HUTCHINGS, D.V.M., M.S., Ph.D.

Lafayette, Indiana

IN A LARGE percentage of dairy cows infected with *Brucella abortus*, the infection becomes established in the udder. The organisms may be eliminated in the mammary secretions for a comparatively long time.

There has been considerable speculation concerning the significance of *Br. abortus* infection in the udder. In the minds of many investigators, there exists considerable doubt that *Br. abortus* produces significant pathologic changes in the mammary tissues of dairy cattle. There is, however, considerable presumptive evidence that the mammary tissues are disturbed, as indicated by a continued high titer of agglutinins in the milk from quarters from which *Br. abortus* has been isolated by direct plating methods and by guinea pig inoculations. There is also evidence to incriminate *Br. abortus* as the cause of inflammatory foci in the tissues of the udder.

It is definitely known that *Brucella suis* occasionally may gain access to the mammary tissues of dairy cattle. In cases where *Br. suis* was transmitted from swine to cattle, it has been found that the organism was present in the milk of such animals and was responsible for multiple cases of brucellosis in human beings.

The apparent relative infrequency of *Br. suis* infection in the bovine animal probably has been responsible for the failure of investigators to study extensively the nature of the infection in this unusual host. At this Station,[1] the problem has been under investigation for considerable time. This report involves a rather detailed study of 10 lactating dairy cows in which *Br. suis* infection was established by introducing a suspension of living *Br. suis* organisms directly into the udder via the teat orifice.

From the Department of Veterinary Science, Purdue University, Lafayette, Ind.

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REVIEW OF LITERATURE

Cooledge[2] found that milk from the udders of Brucella-infected cows had an average cell count several times as high as that from apparently normal cows. He was of the opinion that this high cell count in milk from apparently normal udders was indicative of a "*Bacterium abortus*" infection. The condition, however, was not considered specific. He also concluded that in naturally occurring udder infection the rear quarters were the first to show "*Bacterium abortus*" infection, indicating the genital discharges and switching of the tail to be its source. He thought that the infection could then be carried to the front quarters by the hands of the milkers.

Tweed[3] confirmed some of the findings of Cooledge. He showed that the milk from *Br. abortus*-infected udders had an increase in cell count of more than twice the number found in noninfected udders.

Smith, Orcutt, and Little,[4] in studying the source of agglutinins in the milk of cows, found that when suspensions of "*Bacillus abortus*" were injected into a quarter there was prompt reaction in that quarter manifested locally by swelling of the entire quarter and copious migration of polynuclear leucocytes into the milk. They found that the mastitis produced lasted several weeks. A forequarter that was inoculated with living "*Bacterium abortus*" twenty-three hours before the animal was de-

stroyed showed a very extensive filling of the secretory alveoli with polynuclear leucocytes. The same kind of cells was found in the epithelial and interstitial tissues where the reaction was appreciable in extent. This animal was a tuberculin reactor and also showed hemolytic streptococci in the milk from all quarters. In a second case treated in a like manner, histologic examinations of the exposed quarters and one unexposed quarter showed the alveoli filled loosely with polymorphonuclear cells and to some extent acidophils. The interstitial tissue showed slight infiltrations with lymphoid and plasma cells. In a section from another quarter of the udder, there was found an area where the alveoli were compressed by endothelial cell accumulations. This animal was also a tuberculin reactor and showed hemolytic streptococci in the milk from all quarters. It was impossible to connect the pathologic changes in those cases of experimentally induced brucellosis due to the complication of tuberculosis and streptococcic invasion. Relative to the udder invasion, Smith and co-workers stated, "the invasion of the udder by *Br. abortus* means its presence and multiplication in the milk in the alveoli and ducts and not in the connective tissue stroma. In other words, the residual milk in the alveoli and ducts rather than the tissues appears to be the seat of multiplication."

Runnells and Huddleson[5] reported on inflammatory foci in the mammary tissues of cows infected with *Br. abortus*, although they observed no Brucella in histologic sections. In 1 animal, *Br. abortus* was recovered from one or more quarters on one or more occasions and, at the same time, there was no evidence of other bacteria persisting in the udder. In this 1 cow, they reported finding small foci of lymphocytic cell infiltrations in the interalveolar connective tissue and varying degrees of atrophy. They noted a few lesions of a more acute nature characterized by degenerative and necrotic changes of the epithelial tissues and a purulent exudate in the alveoli. Hyaline degeneration of the subepithelial connective tissue was observed in a few areas along one of the larger lactiferous ducts in the lower portion of the quarters. These workers concluded that there was considerable circumstantial evidence to incriminate *Br. abortus* as the agent which caused the inflammatory foci in the tissues of the udder. No gross lesions were noted in any of the cases discussed.

Sholl and Torrey[6] believed that *Br. abortus* produces definite changes in the udder which may be quite extensive and may lower considerably the efficiency of the organ.

Pederson,[7] working with 8 heifers, fourteen to twenty months old, and exposed to *Br. abortus* two to three months prior to slaughter, found cellular infiltrations which tended to localize in the larger secretory ducts. In 3 of these animals, Staphylococcus cultures were also injected into the teat canal of one quarter, but the changes produced were only temporary. Subsequently,[8] working with 7 heifers during their first lactation, he reported that *Br. abortus* in the udder produced a chronic proliferative inflammation which, although not clinically demonstrable in most cases, not infrequently leads to a lower milk yield of the affected quarters.

Sholl,[9] reporting on histologic examination of the udders of 22 heifers which reacted positively to *Br. abortus* infection by the serum-agglutination test, found evidence of interstitial mastitis to be present in 20 of them, as contrasted to 3 cases of slight to moderate interstitial mastitis in 3 out of 7 heifers not infected with *Br. abortus*. He concluded that udders of heifers may become infected with *Br. abortus* as early as eight months of age and that the lesions of interstitial mastitis, which he considers may result from infection with this organism, may occur in the mammary tissues of nonlactating animals.

Kastli and Saxer,[10] on the basis of work on some 400 individual milk samples sent in for examinations in the course of routine milk control in the Berne district, did not consider that *Br. abortus* infection bears any relationship to catarrhal mastitis or even causes a predisposition to mastitis.

Schlotthauer[11] concluded that, following the elimination of Brucella infection in ten herds of cattle, there was a striking decrease in the number of retained membranes, calf mortality, and cases of clinical mastitis.

Hallman, Sholl, and Delez,[12] reporting on a study of the udders of 15 cows concluded that "*Bacterium abortus*" infection of the udder is associated with pathologic lesions. They found the predominating condition to be a subacute or chronic interstitial mastitis.

Bryan and Meyer[13] found that the quality of milk secreted by *Br. abortus*-infected udders was decreased as compared with that from healthy udders.

EXPERIMENTAL PROCEDURE

Our work was planned to include, in so far as possible, a complete bacteriologic and histopathologic study of the udders and a serologic study of the blood of dairy cattle which had been exposed to living *Br. suis*. All animals used in the trials originated from brucellosis-free herds and individually failed to react to the serologic test for brucellosis.

Ten cows were exposed by either injecting a suspension of living *Br. suis* directly into the milk cisterns or by introducing the organisms just inside the teat sphincter. Exposure was effected by introducing a sterile milk tube into the teat canal after the teat extremity had been

carefully cleansed with 70 per cent alcohol and the suspension of *Br. suis* forced into the milk cistern by means of a 5-cc. syringe attached to the milk tube. Through error, 7 animals used in the trials were not examined bacteriologically for the presence of organisms in the udder prior to initiation of the work. Physically, however, the udder of each animal was free of gross changes commonly associated with udder damage and the milk appeared to be normal.

Following intramammary exposure to *Br. suis*, bacteriologic examinations of the milk were made thrice weekly. The udders were cleaned carefully followed by thorough cleaning of the teat extremity with 70 per cent alcohol. A sample of milk was collected from each quarter into a sterile test tube after several streams were first milked out and discarded. The milk samples were allowed to stand overnight to allow the cream to rise. The following day, the cream was removed with a sterile pipette, placed on tryptose-agar (Difco) plates, and spread with sterile glass rods. The plates were then placed in an incubator at 37 C. for forty-eight hours and examined for the presence of Brucella organisms. At periodic intervals, the suspicious appearing colonies were transferred and examined by the standard methods for identification of *Br. suis*.

Blood samples were collected at weekly intervals and examined bacteriologically for Brucella organisms as well as for serum-agglutination response. The rapid or plate-agglutination test was used with serum dilutions of 1 : 25, 1 : 50, 1 : 100, 1 : 200, and 1 : 400. In several instances, the test tube method was employed to determine titers in excess of 1 : 400. About 60 cc. of blood were drawn aseptically from the jugular vein and cultured for a period of two weeks in 150 cc. of tryptose broth (Difco) during which two subculture platings were made on regular tryptose-agar plates (Difco). Examinations of fetal membranes and colostrum for Brucella organisms were made when possible in cases of parturition during the course of the experiment.

At the time of slaughter, a postmortem examination of the animal was conducted. The udder, internal reproductive organs, popliteal, prefemoral, supramammary, external and internal iliac, mesenteric, gastrohepatic, bronchial, mandibular, cervical, and suprapharyngeal lymph nodes, liver, spleen, heart, kidneys, and lungs were examined for the presence of Brucella. Sections of mammary tissue were removed from three different levels of each quarter and smeared on tryptose-agar (Difco) plates. These same sections of tissue were then fixed in formalin for histopathologic study. In addition, the supramammary lymph nodes were retained for microscopic examination. All tissue sections were stained with hematoxylin and phloxine according to the method described by Mallory.[14]

EXPERIMENTAL DATA

Case 1 (Cow AA).—Case 1 was a grade Guernsey cow in her seventh month of pregnancy when purchased. The animal was exposed by injecting a suspension of approximately 1 billion living *Br. suis* organisms into the milk cisterns of the right front and right rear quarters of the udder. Ten days after such exposure, the blood serum-agglutination titer had risen from negative at a dilution of 1 : 25 to positive at a dilution of 1 : 2,560. Twenty-eight days after exposure, the titer had risen to 1 : 10,240. Blood cultural results for Brucella organisms were negative throughout in this animal. *Brucella suis* was recovered from the milk of the exposed quarters continuously from the time of exposure until slaughter. Forty-two days after exposure, *Br. suis* recoveries were made from the left front quarter and such recoveries continued until slaughter fifty-eight days later. This animal delivered a normal healthy calf. Brucella organisms were not recovered from the fetal membranes. Cow AA was destroyed on the fourth day after parturition for bacteriologic and histologic examination. At the time of slaughter, the blood serum titer was 1 : 2,560. As shown in table 1, *Br. suis* was recovered from the tissues of all four quarters of the udder in addition to the uterus, supramammary, bronchial, suprapharyngeal, and external and internal iliac lymph nodes.

Five days after exposure, the right front and right rear quarters of the udder exhibited gross pathologic changes. The affected quarters showed considerable swelling with firmness and redness indicating an acute inflammation. The external changes were accompanied by changes in the milk ranging from slight flakiness to extreme ropiness, with marked diminution of secretory activity during the height of the inflammatory reaction. This condition continued approximately two weeks after which it gradually subsided.

Microscopic examination of the mammary tissues showed well-marked changes in the right front and right rear quarters. The most prominent inflammatory change appeared to be lymphocytic cell accumulations in the interalveolar connective tissue. A few areas showed lymphocytic accumulations in the alveoli. In these reactive areas, some desquamated epithelial cells were also

observed in the alveoli. The changes were most prominent in the ventral and middle portions of the injected quarters. A marked increase in the interalveolar connective tissue involving several large areas was also noted. With the exception of a small focus of lymphocytic cell infiltration in the interalveolar tissue in the ventral portion of the left rear quarter, little change could be noted in this quarter. The left front quarter showed more marked alterations. The areas affected were most prominent in the ventral and middle portions of the quarter. Foci of lymphocytic cell accumulations were noted in moderate degree in the interalveolar tissue. Accumulations of lymphocytic and polymorphonuclear cells appeared in the alveoli of the reactive areas. There appeared to be some increase in interalveolar connective tissue with concurrent atrophy and distortion of alveoli.

The most important changes noted in the right and left supramammary lymph nodes were apparent increase in the connective tissue elements plus slight lymphoid exhaustion in the germinal centers.

Case 2 (Cow BB).—Case 2 was a grade Holstein-Friesian cow which was in her seventh month of pregnancy when exposed to Brucella. The animal was exposed to *Br. suis* by injecting a polyvalent suspension of approximately 650 million living organisms (laboratory strains 67, 4041, and 1779) into the milk cisterns of the right front and right rear quarters of the udder. Ten days following intramammary exposure, the cow's blood serum-agglutination titer rose from negative at dilution 1:25 to positive at 1:5,120. Eighteen days later, her titer rose to 1:10,240. *Brucella suis* was recovered from the milk of the right front and right rear quarters until the time of slaughter four months later. Three months after exposure, *Br. suis* began to appear in the milk from the left front quarter of the udder.

Two months after exposure, a normal healthy calf was born. Guinea pig inoculation of a suspension prepared from triturated fetal membranes failed to disclose the presence of Brucella organisms. A direct plate count on standard tryptose agar of milk from the right rear quarter made thirty days after exposure revealed the presence of 2,162 living *Br. suis* organisms per cubic centimeter of milk. Blood cultural examinations failed to reveal the presence of *Br. suis* in the blood stream at any time during the course of the experiment. Cow BB was slaughtered approximately four months after exposure to *Br. suis* and examined for the presence of Brucella. At the time of slaughter, the blood serum-agglutination titer had receded to positive at a dilution of 1:5,120. *Brucella suis* was recovered from the tissues of all four quarters of the udder and also from the supramammary lymph nodes, as indicated in table 1.

TABLE 1—Results of Postmortem Bacteriologic Examination of Cows Exposed to Brucella Suis

Cow (No.)	Supramammary l.n.		Udder				Uterus	Gastrohepatic l.n.	Bronchial l.n.	Suprapharyngeal l.n.	Prefemoral l.n.		Iliac l.n.	
	Right	Left	RF	RR	LF	LR					R.	L.	Ext.	Int.
AA	+	+	+	+	+	+	+	—	+	+	—	—	+	+
BB	+	+	+	+	+	+	—	—	—	—	—	—	—	—
CC	+	—	+	+	—	—	—	—	—	—	—	—	—	—
DD	+	—	+	+	—	—	—	+	—	—	—	—	—	—
EE	—	—	—	—	—	—	—	—	—	—	—	—	—	—
FF	—	—	—	—	—	—	—	—	—	—	—	—	—	—
GG	+	—	+	+	—	—	—	—	—	—	+	+	—	—
HH	+	+	+	+	—	—	—	—	—	—	+	+	—	—

+Indicates recovery of *Brucella suis*.

The injected quarters showed considerable swelling and reddening and there was a sharp decrease in milk production beginning the third day after exposure. The milk obtained from the affected quarters exhibited considerable change, as evidenced by extreme ropiness at the height of the reaction. The acute swelling lasted approximately ten days, after which the inflammation gradually receded.

Microscopic examination of the udder showed marked changes in the ventral and middle portions of the right front and right rear quarters although there was also considerable change in the dorsal portions. The changes noted were not uniform throughout the section but were rather

focal in distribution. The predominating changes were subacute or chronic in nature with a marked increase in the amount of interalveolar elements. These consisted of proliferating fibroblasts and leucocytic infiltration. The latter were predominately lymphocytes with a very few polymorphonuclear cells. A hyaline-like or thick serous exudate containing polymorphonuclear cells, lymphocytes, and a few epithelial cells was noted in several alveoli in the affected areas. With the proliferation of interalveolar tissue, there was a corresponding atrophy and occasionally complete disappearance of the alveoli in the reacting areas.

The changes observed in the left front and left rear quarters resembled those in the right half of the udder. The predominating change was focal interstitial infiltration by lymphocytic cells with increase in connective tissue.

No microscopic changes were observed in the supramammary lymph nodes.

Case 3 (Cow CC).—Case 3 was a grade Holstein-Friesian cow in her sixth month of pregnancy when exposed to Brucella organisms. This animal was first exposed by smearing living *Br. suis* on the abraded skin over the left front and left rear quarters. The procedure was repeated twice at two-week intervals. *Brucella suis* was not recovered from the milk at any time after these exposures. A slight blood serum-agglutination response was elicited, being positive at a serum dilution of 1 : 50. The agglutination titer became negative within three months. This cow delivered a full-term normal healthy calf. Bacteriologic examination of the colostrum at the time of calving failed to disclose the presence of living Brucella organisms. Approximately five and one-half months after the previously mentioned cutaneous exposure, she was again exposed by injecting a polyvalent suspension of approximately 2 billion living *Br. suis* organisms into the right front and right rear quarters of the udder. The suspension of organisms used included two laboratory strains of *Br. suis* plus *Br. suis* which had been recovered from case 2. *Brucella suis* recoveries from the injected quarters continued from the day after exposure until slaughter eleven days later. At the time of slaughter, the blood serum-agglutination titer had risen to positive at a dilution of 1 : 2,560. *Brucella suis* was recovered from both right quarters and also from the right supramammary lymph node, as shown in table 1.

No change in the appearance of the udder was noted following cutaneous exposure to *Br. suis*. However, following reëxposure by the injection of living *Br. suis* into the teat canals, there was a very marked and severe reaction by the mammary tissue which resulted in pronounced swelling and reddening of the exposed quarters. The secretions of one of the exposed quarters assumed the nature of a serosanguinous secretion. At the time of slaughter, the affected quarters were very much altered grossly.

Microscopic examination of the right front and right rear quarters of cow CC showed pronounced changes. There were large areas of infiltrations of lymphocytes in the interalveolar spaces. Although the lymphocyte was the predominating infiltrative cell, some polymorphonuclear and eosinophilic cells were also present. Lymphocytes, polymorphonuclear, and epithelial cells were also noted in the alveoli of the reactive areas. Some alveoli showed separation of the epithelial cells from the basement membrane with some exfoliation into the alveoli. Proliferative changes in the interalveolar connective tissue were quite pronounced in many areas.

The left front and left rear quarters showed a few small areas of interalveolar lymphocytic cell infiltrations. Aside from these, no changes were noted.

The right supramammary lymph node showed an increase in the lymphocytic elements of the germinal centers. No changes were noted in the left supramammary lymph node.

Case 4 (Cow DD).—Case 4 was a grade Guernsey cow in her second month of pregnancy when exposed to *Br. suis*. This animal was exposed, as in the previous case, by smearing a culture of living *Br. suis* (laboratory strains 4041, 67, and 64) on the abraded skin of the left front and left rear quarters of the udder. The procedure was repeated twice at two-week intervals. A slight blood serum-agglutination response was elicited, being positive at a serum dilution of 1 : 25 eleven days after initial exposure. This response was transient in nature and receded to nega-

tive within four months. Brucella organisms were not recovered from the milk following these exposures. Parturition was normal. Placental tissues were not obtained for culture or for guinea pig inoculation. Guinea pig inoculation with colostrum failed to reveal the presence of Brucella organisms. Approximately one month after calving, this animal was exposed by introducing a polyvalent suspension of Brucella organisms into the teat canal of the right front and right rear quarters. Included in the suspension was a strain of *Br. suis* which had been recovered from the milk of cow FF. Seven days after exposure, cow DD was destroyed for bacteriologic and histopathologic study. At the time of slaughter, no blood serum-agglutination response had been elicited. *Brucella suis* was recovered from all levels of the exposed quarters sampled. In addition, it was recovered from the right supramammary and gastrohepatic lymph nodes, as shown in table 1.

No change in the gross appearance of the udder was noted following cutaneous exposure to *Br. suis*. Following reëxposure to Brucella by introducing a living culture of *Br. suis* into the teat canal, there was a very marked and severe reaction in the exposed tissues. The affected quarters showed considerable swelling and reddening. The secretory activity ceased temporarily and the animal showed considerable discomfort, going partly off feed. At the time of slaughter, the reaction was perhaps at its height. The affected quarters of the udder were considerably larger than the unexposed quarters.

Microscopically, the most prominent change in the right front and right rear quarters appeared to be infiltration of lymphocytic cells into the interalveolar connective tissue. A number of alveoli were

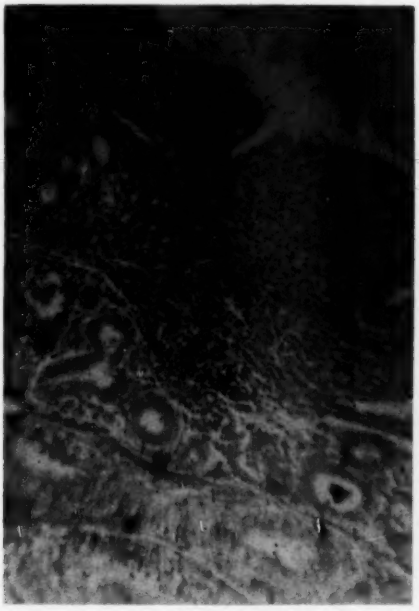

Fig. 1—Section taken from middle portion of right front quarter of cow CC showing accumulation of lymphocytic, polymorphonuclear, and eosinophilic cells near a blood vessel. Note distortion of alveoli and interalveolar fibrosis and slight amount of intra-alveolar exudate containing lymphocytes and desquamated epithelial cells. x 150.

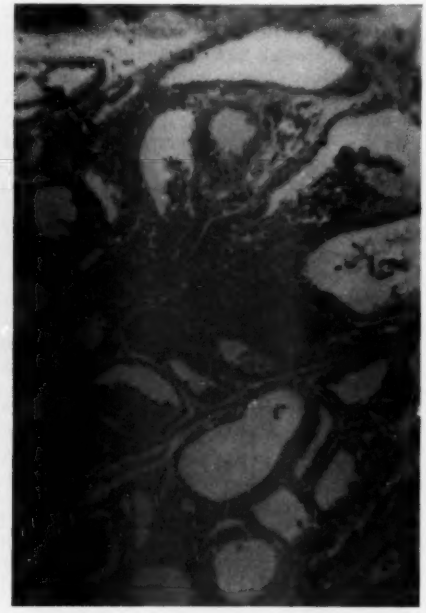

Fig. 2—Section taken from the dorsal portion of the right front quarter of cow DD showing interalveolar infiltration by lymphocytes and polymorphonuclear cells in addition to proliferation of fibroblasts. Note leucocytic infiltration into alveoli with epithelial separation from basement membrane and exfoliated epithelial cells in the alveoli. x 150.

filled with a serous exudate containing lymphocytes, polymorphonuclear leucocytes, and some desquamated epithelial cells, as shown in fig. 2. The changes appeared uniformly throughout the affected quarters. Several infiltrated foci containing proliferating fibroblasts were also noted, as shown in fig. 3.

No pathologic changes were observed in the left quarters of the udder.

The right supramammary lymph node showed a slight increase in the lymphocytic elements of the germinal centers. The corresponding node from the left side was misplaced and hence not available for microscopic examination.

Case 5 (Cow EE).—Case 5 was a grade Holstein-Friesian cow not pregnant when put on experiment. Prior to exposure, milk was examined for the presence of organisms commonly considered as causes of mastitis in order to eliminate the possibility of a mastitis being produced by a non-Brucella pathogen. Gross physical examination also failed to disclose mastitis in any quarter. Examination of the milk for pathogens was repeated five times during a two-week period. Exposure was effected by introducing 290 million living *Br. suis* organisms into each right quarter. Seven days after exposure, the serum-agglutination response had risen to positive at dilution 1:50. Six days later, cow EE reacted positively at a serum dilution of 1:400. Brucella was not recovered from the blood during the course of the experiment. Exposure was followed by extreme swelling of the affected quarters, with almost complete cessation of milk flow for a period of five days. The milk from this cow was fed to 5 pigs all of which became infected with *Br. suis*. At the time of slaughter five months later, Brucella was not recovered from cow EE.

Microscopically, the changes in the right quarters were similar to those seen in the udder of cow DD. No pathology was noted in the left half of the udder or in the supramammary lymph nodes.

Case 6 (Cow FF).—Case 6 was a grade Holstein-Friesian cow which, at the time of exposure to *Br. suis*, was in her fifth month of pregnancy. Exposure was by introduction of a polyvalent suspension of living *Br. suis* (laboratory strains 1779, 4041, and 67) organisms directly into the teat canals of the right front and right rear quarters. Approximately 50 million organisms were injected into each quarter. This animal delivered a normal full-term calf. Direct cultural examination and guinea pig inoculation of colostrum and fetal membranes failed to disclose the presence of Brucella organisms. Twelve days after exposure, the blood serum titer had risen to positive at 1:400. Twenty months after exposure, this animal was destroyed and her tissues examined bacteriologically and histopathologically.

Within five days after exposure, the injected quarters exhibited considerable swelling and tenderness, indicating an acute inflammation. The milk was grossly altered ranging from flakiness to a ropy thick secretion. After the initial swelling, the mastitis subsided and the udder was approximately normal within a month following exposure. Palpation of the exposed quarters several months after exposure revealed an increase in connective tissue in the exposed quarters.

Fig. 3—Section from dorsal portion of right front quarter of cow DD showing cellular infiltration plus pronounced increase in interalveolar connective tissue. x 712.

Microscopic examination of the right front quarter showed considerable increase of interstitial connective tissue with some atrophy and distortion of the alveoli. Focal areas of interstitial lymphocytic infiltration were also noted. The changes seemed to be fairly evenly distributed throughout the different levels of the quarter examined. The right rear quarter showed much the same changes except that there appeared to be relatively more fibrotic areas especially in the dorsal portions of the quarter.

In general, the left side of the udder was relatively free of pathologic changes. A very few small areas of lymphocytic infiltration were noted in a section obtained from the middle portion of the left rear quarter.

Both supramammary lymph nodes appeared to have a relative increase in connective tissue. The cortical septums appeared slightly thickened. A moderate degree of lymphoid exhaustion was noted, especially in the right supramammary lymph node.

Case 7 (Cow GG).—Case 7 was a grade Holstein-Friesian purchased as a pregnant heifer. Several preëxposure examinations of the milk were made for organisms which might be incriminated in mastitis. These examinations failed to reveal the presence of any bacteria commonly accepted as causes of mastitis.

About one month after calving, the cow was exposed to brucellosis by introducing a suspension of living *Br. suis* directly into the right front and right rear quarters through the teat canal. Approximately 2 billion organisms were introduced into each quarter. On the third day after exposure, the animal became very depressed and failed to eat well for two days during the height of the reaction. Ten days after exposure, the animal was killed for bacteriologic and histopathologic examination. *Brucella suis* was obtained in pure culture from the exposed quarters of the udder and also from the right and left prefemoral and right supramammary lymph nodes, as can be seen in table 1.

The right half of the udder exhibited considerable swelling, reddening, and tenderness with the complete cessation of secretion by the fifth day after exposure. On postmortem examination, the injected quarters were edematous and considerably larger than the unexposed quarters.

Microscopically, foci of interalveolar lymphocytic and polymorphonuclear cell infiltrations were numerous throughout the right front and right rear quarters. Proliferation of fibroblasts was noticeable, being most marked in the rear quarter. Lymphocytic and polymorphonuclear cell infiltrations into the alveoli with some desquamation of epithelial cells was a constant finding. Several alveoli contained a serous-like exudate and were irregular in form due to interalveolar pressure.

No pathologic changes were noted in the left front and left rear quarters. There seemed to be some increase in the relative number of lymphoid cells in the right supramammary lymph node. Aside from this, there were no changes observed.

Case 8 (Cow HH).—Case 8 was a grade Holstein-Friesian purchased as a pregnant heifer along with cow GG. Several preexposure milk examinations were conducted on this animal to rule out the possibility of the presence of non-Brucella pathogens.

TABLE 2—Results of Blood Examinations of

Cow (No.)	Route of exposure	Agglutination titer																			
		10					14					28					35				
AA	Teat canal	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
BB	Teat canal	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
CC	Teat canal	+	+	+	+	+*															
DD	Teat canal	—	—	—	—	—!															
EE	Teat canal	+	+	+	P	T	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FF	Teat canal	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	P
GG	Teat canal	P	T	—	—	—:															
HH	Teat canal	+	+	+	+	+*															
II	Teat orifice	—	—	—	—	—	—	—	—	—	—	T	—	—	—	—	+	P	T	—	—
JJ	Teat orifice	—	—	—	—	—	—	—	—	—	—	T	—	—	—	—	+	+	+	+	+

* = removed for slaughter. ! = destroyed on the seventh day. : = sold for slaughter.
+ = complete agglutination. P = partial agglutination. T = trace of agglutination.

Such test failed to disclose the presence of the commonly accepted mastitis-producing microörganisms. Approximately one month following parturition, exposure was effected by injecting a suspension of living *Br. suis* directly into the teat canal of the right front and right rear quarters of the udder. The strain of Brucella used was from a hog which had been exposed to *Br. abortus* and from which *Br. suis* had been recovered. The animal showed no systemic symptoms and remained on feed. She was destroyed on the tenth day after exposure and examined bacteriologically. As in the previous case, *Br. suis* was recovered in pure culture from all levels of the right front and right rear quarters. As can be seen in table 1, *Br. suis* was also recovered from the supramammary and right and left prefemoral lymph nodes. At the time of death, her blood serum-agglutination titer was 1:400.

The exposed quarters did not exhibit the extreme swelling observed in case 7. On postmortem examination, however, the injected quarters exhibited considerably more swelling than was apparent in the living subject.

Microscopically, the pathologic changes observed were uniformly distributed throughout the exposed quarters. Many foci of lymphocytic and polymorphonuclear cell infiltrations were noted in the interalveolar tissue. Proliferation of fibroblasts in several of these areas was rather marked. In the reactive foci, there were alveoli filled with a serous-like exudate containing lymphocytes and desquamated epithelial and polymorphonuclear cells. In several areas, there was a high proportion of eosinophils among the infiltrating cells.

With the exception of one small focus of interstitial lymphocytic cell infiltration in the lower portion of the left rear quarter, no pathologic changes were observed in the left side of the udder.

There seemed to be an increase in lymphocytic cell concentration in the germinal centers of both supramammary lymph nodes. No other changes were observed.

In order to check on the suspending fluids used in cases 7 and 8, a normal first-calf heifer was injected with sterile saline and distilled water (the suspension mediums) in the right front and right rear quarters, respectively. No changes were observed in the mammary gland of this animal following such treatment.

Cases 9 and 10 (Cows II and JJ).—Cases 9 and 10 were grade Guernsey cows not pregnant at the time of exposure to Brucella. These animals were exposed by introducing a suspension of living *Br. suis* into the open teat orifice of the right front and right rear quarters to determine if the infection would ascend into the secreting mammary tissues. Active infection was established following the second exposure. Both animals showed blood serum-agglutination responses indicative of infection, and *Br. suis* was recovered from the milk of each of the exposed quarters for relatively long periods of time. Postmortem examinations were not conducted on these animals.

In order to facilitate ease of interpretation, the results of the blood examinations of the cows exposed to *Br. suis* by the intramammary route have been grouped together in tabular form (table 2). Note that consistently high serum-agglutination titers developed as a result of exposure to *Br. suis*. Brucella was not recovered from the blood of any of the animals. Cow DD

Cows Exposed to Brucella Suis by Intramammary Route

| (days after exposure) | | |

was destroyed shortly after exposure and probably had not had sufficient time to develop a blood serum-agglutination response.

DISCUSSION

Under the conditions of these trials, it has been shown that *Br. suis* infection when localized in the udder was responsible for acute bovine mastitis. Three of the animals were examined carefully, both prior to and following exposure to *Br. suis*, for organisms other than Brucella that might be incriminated as responsible for the changes observed. In view of the fact that organisms other than Brucella or other causes of mastitis such as trauma were not found, it appears that *Br. suis* was responsible for the acute mastitis produced in the experimental animals. The acute mastitis was manifest by pronounced swelling of the affected parts of the udder with tenderness and reddening of the infected areas. Milk from affected quarters was definitely altered, ranging from flakiness and ropiness to a secretion resembling blood serum more than milk. The acute inflammatory reaction gradually subsided and was followed by increased connective tissue in the reactive foci. There is evidence that the changes are progressive in nature, involving the parenchymatous tissues first and the interstitial tissue later. In view of these results, the possible role of Brucella in causing udder damage should be given careful attention.

The results obtained here also indicate that, in the type of exposure employed, *Br. suis* is capable of invading bovine tissue other than the mammary structures.

SUMMARY

1) Under the conditions of the experiment reported here, it has been demonstrated that *Brucella suis* is capable of causing brucellosis in cattle following intramammary exposure. Active infection of pregnant cattle with *Br. suis* did not result in premature expulsion of fetuses.

2) In such cases of active infection resulting from exposure to *Br. suis* via the mammary tissues, acute cases of bovine mastitis developed with the elimination of living *Br. suis* in the milk from affected quarters. Such infection resulted in extreme inflammatory reactions in the exposed quarters with concurrent gross alterations of the mammary secretions and acute symptoms in the cases of marked mastitis.

3) Microscopically, the pathologic changes were characterized by focal infiltrations of lymphocytic and polymorphonuclear cells both interstitially and into the alveolar sacs along with proliferations of fibroblasts and some desquamation of alveolar epithelial cells.

4) Intramammary exposure of cattle to *Br. suis* resulted in blood serum-agglutination responses indicative of infection. At times, the responses reached a titer of positive at a serum dilution of 1: 10,240.

5) Postmortem examination of the animals for *Br. suis* resulted in recovery of the organisms from lymph nodes in widely scattered parts of the body.

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The Use of Ether-Killed Brucella Abortus in Saline-in-Oil Emulsion in a Herd of Cattle with Brucellosis

I. LIVE, V.M.D., Ph.D., and A. G. DANKS, B.S., D.V.M.

Philadelphia, Pennsylvania

IT WAS previously reported[1] that ether-killed *Brucella abortus* combined with adjuvants had proved efficacious in producing resistance in guinea pigs against challenge with virulent *Br. abortus*. Subsequently, an opportunity was afforded to study the effect of ether-killed *Br. abortus* in cattle when an outbreak of brucellosis occurred in a herd which belongs to the University of Pennsylvania. This herd of Guernsey cattle had been accredited for a number of years up to May, 1948, when 1 cow was reported positive in an official state test for brucellosis. The introduction of the infection into this herd has not been satisfactorily explained, and, despite the immediate removal of the positive animal and the instituting of necessary precautions, the disease spread in the herd of approximately 100 cows and heifers. All animals above 6 months of age were tested monthly, and in one year 24 head of cattle were disposed of as reactors, 10 of which had aborted.

Because of the extensive infection, calfhood vaccination with strain 19 vaccine was initiated in January, 1949. In May, 1949, it was decided to vaccinate the older heifers and the cows as well. The present report deals with the study of this group in which ether-killed *Br. abortus* combined with falba and mineral oil was used on the one hand, and strain 19 vaccine on the other, in order to compare the antigenicity, and if possible the immunizing property, of these two substances in cattle.

MATERIALS AND METHODS

Status of Herd Under Study.—At the time of initiation of this study in May, 1949, the entire herd was tested for brucellosis. One cow was found positive, and 1 animal gave a suspicious reaction to the agglutination test. Both animals were disposed of and 73 females of breeding age (32 cows and 41 heifers) were used in the experiment. The animals were divided into the following three groups: group A consisting of 28 animals which received strain 19 vaccine (3 animals of this group which had been more than 5½ months pregnant in May, 1949, were not injected with strain 19 vaccine until about 1 month after parturition); group B consisting of 27 animals which were injected with ether-killed *Br. abortus* combined with falba and mineral oil; and group C consisting of 18 animals which were left as controls. After removal of 2 heifers from each group for challenge purposes, as discussed below, group A was composed of 26 animals, group B of 25, and group C of 16 animals. The cows in the milking herd were allowed to intermingle, but whenever possible they were isolated at the time of parturition. The heifers used in this study had been separated from the herd at weaning time. Some of them were brought back to the main farm during early pregnancy, while others were not returned until they were ready to freshen. Group A contained 11 heifers of which 7 were on the main farm at the time of vaccination; group B contained 14 heifers of which 9 were on the main farm at the time of vaccination; and group C contained 10 heifers of which 6 were on the main farm in May, 1949. (The remaining 6 heifers were used in a challenge experiment.)

Pregnancy Data.—Table 1 shows the pregnancy status of the animals at the time of vaccination. It will be noted that 8 animals in group A were in the fifth or sixth month of gestation (the animal furthest along in gestation was 5½ months pregnant), and 8 animals in group B were in the fifth to seventh month of gestation (the animal furthest along in gestation was almost 7 months pregnant) at the time of vaccination. This plan was adopted, despite the fact that administration of strain 19 vaccine to cattle beyond the fourth month of gestation is considered inadvisable, in order to test the degree of safety of administering large doses of ether-killed vaccine to animals of such status in comparison with a similar group treated with strain 19 vaccine. The number of controls beyond the fourth month of gestation at the beginning of the experiment was proportionately smaller than that in the other two groups.

From the School of Veterinary Medicine, University of Pennsylvania, Philadelphia.

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Immunizing Substances.—The strain 19 vaccine used was a commercial, lyophilized product which, according to plate counts carried out at the time of vaccination, contained 12 billion live organisms per milliliter of suspension. The animals of group A were injected once subcutaneously with 5 ml. of this vaccine.

Recently isolated CO_2-sensitive strains of *Br. abortus* were used in the preparation of the whole cell vaccine killed with ethyl ether. The cultures were grown on tryptose-agar slants under 10 per cent CO_2 tension for forty-eight hours, and the growth on each slant was washed off with 4 ml. of 0.85 per cent NaCl. The physiologic saline suspension of cells was removed and centrifuged at high speed for one hour. Then, the supernatant fluid was poured off and the sediment was washed once in 0.85 per cent NaCl. Finally, the sediment was resuspended in one sixth of the original quantity of 0.85 per cent NaCl, and about 50 per cent by volume of ethyl ether was added. Prior to the addition of ether, a small quantity of the material was removed and diluted, and colony plate counts were made to determine the number of Brucella cells in the original suspension. The suspension was kept in the refrigerator until used.

The saline-in-oil emulsion of the vaccine was prepared according to the technique of Freund and McDermott.[2] The falba and the mineral oil were sterilized in the autoclave. Then, one part of the desired dilution of a saline suspension of ether-killed Brucella was emulsified by means of an electric stirrer in one part of melted falba; to this, one part of mineral oil was added, with continued agitation. The animals of group B were injected subcutaneously three times at fourteen-day intervals with ether-killed *Br. abortus* combined with falba and mineral oil, the first injections being given at the time of vaccination of group A with strain 19 vaccine. In each instance, the animals received two simultaneous injections of 1.5 ml. which contained a total of approximately 100 billion Brucella cells. The three double injections were made one on each side: anterior to the shoulder, posterior to the shoulder, and above the shoulder, respectively. Six months after administration of the last of three doses of killed vaccine, 25 animals of this group received another injection of ether-killed *Br. abortus* combined with falba and mineral oil (the remaining 2 animals had in the meantime been used in a challenge experiment). At this time, a 3-ml. dose containing approximately 150 billion ether-killed *Br. abortus* organisms was injected subcutaneously into one site on the neck.

Blood-Agglutination Tests.—All animals under study were bled twice at two-week intervals after the initial date of vaccination, and once a month thereafter, for the purpose of carrying out an agglutination test. The standard tube method was used, and in each instance the end titers of the blood serum was determined.

Whey-Agglutination Tests. — Approximately every two weeks, a composite sample of milk was obtained from individual lactating animals at the time of a regular afternoon milking. Sterile vials were filled with about 25 ml. of milk and were brought to the laboratory. After the cream had risen, 5 ml. of the skim portion of each sample was transferred to a separate test tube, and a small quantity of rennet powder was added to each tube. Then, the tubes were placed in an incubator for one to two hours, after which they were centrifuged to cause separation of the whey from the curd. In carrying out the agglutination test, the standard tube method was used, and the end titers were determined, beginning with a 1 : 5 dilution of the whey.

Culture Studies.—The farm is located 30 miles from the laboratory and, because of the distance, it was not always feasible to obtain specimens frequently and promptly. However, whenever possible, aborted fetuses were examined for the presence of Brucella by culturing the abomasum contents and the tissues of various organs on tryptose agar. Cultures were made in duplicate on tryptose-agar plates, one set of plates being incubated under 10 per cent CO_2 tension and the other under ordinary atmospheric conditions. When the Brucella required CO_2 for growth on primary isolation, it was considered a field strain of organisms. On the other hand, if the Brucella grew well under ordinary atmospheric conditions when first cultured from the tissues, it was assumed to belong to the strain 19 group of organisms. Quarter samples of colostrum from animals which aborted, and subsequently cream from milk samples, were also cultured.

In addition to the culturing of the various tissues, each of 2 guinea pigs was injected subcutaneously with 2 ml. of a tissue emulsion of liver and spleen, and each of 2 guinea pigs was injected with 2 ml. of abomasum contents from the fetuses brought to the laboratory. About five weeks after inoculation, the guinea pigs were bled for the purpose of carrying out an agglutination test; then they were sacrificed and their spleens were cultured.

All Brucella cultures were identified by preparing an antigen from a transfer of the isolated organisms and carrying out an agglutination test with a known positive and a known negative Brucella serum.

Challenge Experiment.—In addition to the study of the two vaccines in cattle under field conditions, 2 heifers from each group were challenged with virulent *Br. abortus* five months after vaccination. The 6 animals ranged from 21 to 31 months in age, and they were in the fifth month of gestation at the time of challenge. The organisms used in the

challenge dose were from strain 2308 of *Br. abortus* (aërobic) which was obtained in lyophilized form from the Bureau of Animal Industry, Beltsville, Md., through the courtesy of Dr. C. A. Manthei. The lyophilized culture was suspended in a small volume of sterile saline and subcultured once on a tryptose-agar slant from the growth of which the challenge dose was prepared. Each heifer received 0.1 ml. of a saline suspension of the organisms, which was instilled into the conjunctival sac and which contained 166 x 10^4 Brucella cells, according to plate counts carried out at the time of challenge. Guinea pigs were injected at the same time with various dilutions of the same suspension, and it was found that as few as 17 organisms (the smallest dose used) were sufficient to produce infection. Manthei and Carter[6] reported that 370 x 10^6 and 740 x 10^6 organisms of this strain caused infection in 77 per cent and 89 per cent of unvaccinated animals, respectively.

From the time of challenge until after parturition, the temperature of each animal was recorded daily, morning and evening, in order to ascertain any thermal reaction resulting from a possible infection. Blood samples were obtained weekly up to several months after parturition in order to carry out an agglutination test on the serums. At parturition, placenta and colostrum were cultured on tryptose agar, and suspensions of placenta and colostrum were injected subcutaneously in 2-ml. quantities into different groups of guinea pigs for the purpose of detecting any Brucella. In the case of an abortion, the same procedure was followed, but in addition the fetus was included in the culture- and animal-inoculation studies. After parturition or abortion, quarter samples of milk were obtained weekly for several months. The cream was cultured for the presence of Brucella, and an agglutination test was carried out on the milk whey.

RESULTS

The results reported are those obtained in the course of a year after vaccination.

Blood Serum-Agglutination Reactions as a Result of Vaccination.—The animals used were negative to the agglutination test for brucellosis at the beginning of the study. They were tested two weeks after the initial injections of the vaccines, again two weeks later, and once a month thereafter. All control animals remained negative to the agglutination test. Group A showed a mean agglutinin titer of 1 : 1,128 two weeks after administration of the vaccine. The titers receded rather rapidly so that two months after vaccination the mean agglutinin titer of group A was 1 : 310. From this time, the titers showed only slight fluctuations. The animals in group B, on the other hand, had a mean agglutinin titer of 1 : 620 two weeks after injection of the first dose of ether-killed vaccine. The titers rose somewhat during the next two months and remained at about the same level for

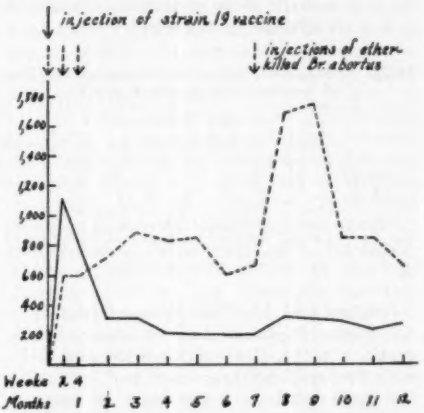

Fig. 1—Agglutinin titers (mean) in cattle after injection of strain 19 vaccine (——), and after injections of saline-in-oil emulsion of ether-killed Brucella abortus. (— — — —)

the following two months. Then, the titers declined somewhat. However, the administration of another dose of ether-killed *Br. abortus* combined with falba and mineral oil, six months after the first series of vaccine injections, was followed by a sharp rise in agglutinin production which was sustained for about two months. The mean agglutinin titers of group A and group B are presented in fig. 1.

Whey-Agglutination Tests.—All lactating animals were negative to the agglutination test in a 1 : 5 dilution of the whey at the time of vaccination. After vaccination, most animals in group A developed whey titers ranging from 1 : 5 to 1 : 80. However, the titers showed fluctuations within the same animal in the course of the study, and in some animals low concentrations of agglutinins still were present in the whey one year after vaccination. Five animals of group A showed no agglutinins in a 1 : 5 dilution of the whey throughout the period of study, although the blood serum agglutinin titers of these animals ranged

from 1 : 200 to 1 : 800. On the other hand, all animals of group B showed agglutinins in the milk whey at some time after vaccination. The titers varied between 1 : 5 and 1 : 160, and here also fluctuations in agglutinin concentration were observed within the same individuals. The agglutination tests carried out on the milk of the control animals were consistently negative in a 1 : 5 dilution of the whey.

TABLE 1—Pregnancy Status of Animals at the Time of Vaccination of Groups A and B

	Month of pregnancy								
	Open	1st	2nd	3rd	4th	5th	6th	7th	9th
Group A*	13	3			1	3	5		
Group B	10	3	2		4	3	4	1	
Group C	9		1	1	4	1	1		1

*Three additional animals which were more than five and one-half months pregnant at the time of vaccination of this group were not injected with strain 19 vaccine until after parturition.

Calving and Abortion Data.—Group A.—As shown in table 2, 8 animals of this group aborted, 2 of which (495 and 549) were first calf heifers which had been with the main herd from the time of vaccination. In addition, another animal (503) freshened about six weeks prematurely, and the calf died within twenty-four hours. The first animal to abort was cow 478, which dropped a 6½-month-old fetus twenty-three days after vaccination. The fetal tissues were negative on cultures, but no further studies could be made because the animal was disposed of three days after the abortion. This animal showed the highest agglutinin titer (1:3,200) in group A two weeks after vaccination. The fetuses aborted by cows 388, 430, and 437 yielded Brucella on cultures which grew only when incubated under 10 per cent CO_2 tension. Therefore, it was concluded that these represented a field strain of *Br. abortus*. In the case of cow 388, which had been five and one-half months pregnant at the time of vaccination and which aborted one month later, it would be reasonable to assume that she was in the incubation stage of the disease at the time of the vaccination. Cow 430, which had been bred fourteen days before vaccination, aborted 238 days after administration of the vaccine; and cow 437, which had been in her fifth month of pregnancy at the time of vaccination, aborted 126 days later. In these 2 cases, the infection may have taken place shortly before or after vaccination, as is suggested by the fact that the period between vaccination and abortion was rather long. Thomsen[4] found that the average incubation period in animals infected at the time of service was 225 days and that the average incubation period in animals infected during the fifth month of pregnancy was eighty days. Heifer 549, which had been vaccinated in her fifth month of gestation, aborted 109 days later, and cultures of the fetal tissues and of the colostrum yielded Brucella which grew readily under ordinary atmospheric conditions. Furthermore, guinea pigs injected with fetal material did not show any agglutinins in their blood serums at the time of autopsy, and their tissues did not yield any Brucella on cultures. Therefore, since the organisms did not require CO_2 for isolation and appeared nonpathogenic for guinea pigs, it was concluded that the infection was due to strain 19 of *Br. abortus*. Heifer 495 and cow 387, which had been vaccinated in their sixth month of pregnancy, aborted

TABLE 2—Abortion Data

Animal (No.)	Month of pregnancy at time of vaccination	Aborted days after vaccination	Results of cultures of fetal tissues
	Group A†		
478	Fifth	23	—
388	Sixth	31	+•
495	Sixth	99	0
549	Fifth	109	+••
387	Sixth	111	0
437	Fifth	126	+•
430	First	238	+•
480	Open	212 (twins)	—
	Group B		
444	Second	153	+•
	Group C (nonvaccinated controls)		
427	Fourth	108 (twins)	—

†One animal of this group (cow 503) calved about six weeks prematurely, and the calf, which was born weak, died within twenty-four hours.

Results of cultures: + = positive; — = negative; 0 = not cultured; • = isolated CO_2-sensitive strain of Brucella; •• = isolated aërobic strain of Brucella.

ninety-nine days and 111 days after vaccination, respectively. The abortions took place during the night in summer, and the fetuses were badly decomposed so that they could not be cultured. Milk samples obtained from these animals on several occasions were negative to Brucella on cultures. However, 1 of them (heifer 495) showed a twofold increase in the agglutinin concentration of the serum soon after abor-

tion. This might be indicative of infection, since Metzger and Shuart[5] found that infection in vaccinated cattle is accompanied by an increase in a stabilized postvaccination titer. A similar fourfold increase in the agglutinin titer was observed after the premature parturition of cow 503, which had been vaccinated in her sixth month of pregnancy. The calf was born weak and died within twenty-four hours. Cow 480, which had been bred forty-five days after vaccination, aborted a pair of twins in her sixth month of pregnancy. Cultures of the fetuses, colostrum, and milk were negative to Brucella, and the agglutinin titer of this animal did not show any deviation from the usual postvaccination pattern. Altogether, 16 animals of group A produced normal calves and 1 heifer has not freshened yet.

Group B.—In this group, 1 cow which had been bred forty-one days before vaccination aborted 153 days after administration of the first dose of vaccine. The *Br. abortus* isolated from the fetus required CO_2 for growth on culture mediums. Therefore, it was concluded that the organisms represented a field strain of Brucella. According to Thomsen's[4] findings with respect to the length of incubation periods in brucellosis, this animal might have become infected before or soon after vaccination. Of the remaining 24 animals, 22 calved normally and 2 have not freshened yet.

Group C.—One animal of this group aborted a pair of twins in her eighth month of pregnancy. However, since no Brucella was isolated from the fetuses and the cow remained negative to the agglutination test for months, the abortion can not be attributed to Brucella infection. Twelve animals in this group calved normally, 2 animals were sold because of breeding difficulties, and 1 cow is still pregnant.

Local Reaction at the Site of Injection of Vaccine.—The majority of animals in group A developed swellings at the site of injection of the vaccine. These swellings reached their maximum size, ranging up to 10 cm. in diameter, within a week or two; then they receded gradually and disappeared or were reduced to a much smaller size within six to eight weeks. In group B, also, most of the animals showed swellings at the site of one or more of the injections. In general, these swellings were smaller than those observed in group A, but in some instances tended to persist for months.

Challenge Experiment.—The 6 heifers, 2 from each group, used in this experiment had been weaned and separated from the main herd before May, 1948, when the first case of brucellosis was diagnosed. Therefore, it may be reasonably assumed that they had no contact with infection until they were artificially exposed to *Br. abortus*, at which time they were segregated in a barn some distance from the main herd. At the time of challenge, the 2 control heifers were negative to the agglutination test in a 1 : 25 dilution of their serums, the 2 heifers injected with ether-killed vaccine had titers of 1 : 200 and 1 : 800, respectively, and the 2 heifers which had received strain 19 vaccine had titers of 1 : 100 and 1 : 200, respectively. No appreciable changes were noticed in the agglutinin titers of the vaccinated animals after challenge throughout the period of gestation and for several months after parturition. On the other hand, 1 of the control animals showed agglutination in 1 : 200 dilution of the serum three weeks after challenge, and the titer increased steadily until it reached a peak of 1 : 3,200 two weeks after abortion. This animal aborted sixty-eight days after exposure, and an aërobic strain of *Br. abortus* was isolated from the fetus, from the colostrum, and on subsequent, repeated culturing of milk (the culture used for challenge was aërobic strain 2308). A marked rise in body temperature was noted in this animal fifty-two days after challenge. The temperature recorded in the morning was 103 F., and it increased to 106.9 by evening. The next morning the temperature was still 105.8, but it was reduced to 103.8 by evening and was normal after that. The marked elevation in body temperature observed in this heifer only may have been associated with a systemic reaction to the infection, since no other explanation could be found for it and since she was the only animal in the group which was proved to be infected. The other control animal remained negative to the agglutination test throughout the period of pregnancy, calved normally, and was negative to Brucella upon culturing and guinea pig inoculation of placenta and colostrum. Milk samples obtained weekly for two and one-half months after parturition did not yield Brucella on cul-

tures, and the animal remained negative to the blood serum-and whey-agglutination tests. It should be mentioned that this was the only 1 of the 6 heifers in which oozing of the bacterial suspension from the conjunctival sac was noticed at the time of challenge. Thus, it would appear that this heifer had not been exposed to the same number of Brucella organisms as the other 5 heifers.

The 2 heifers injected with ether-killed vaccine and the 2 injected with strain 19 vaccine calved normally, and no Brucella was isolated from the placenta, colostrum, or milk samples which were cultured weekly for two and one-half to three months after parturition.

DISCUSSION

In this preliminary study, it was attempted to investigate the antigenicity and the immunizing quality of a saline-in-oil emulsion of ether-killed *Br. abortus* in cattle, as compared with those properties of strain 19 vaccine. Eichhorn *et al.*,[6] in studying the effect of various excipients upon the behavior of strain 19 vaccine, did not find that they had any significant influence upon the agglutinin response in rabbits and cattle. On the other hand, various investigators[7-11] have observed that with the use of falba and mineral oil the antigenicity of an inoculum is markedly prolonged, as judged by the duration of the titer resulting from such injections. Similar results were observed in guinea pigs[1] when ether-killed *Br. abortus* suspensions incorporated into falba and mineral oil were used, and again in the present study when such a saline-in-oil emulsion of ether-killed vaccine was administered to cattle. Here, the results of injection of live strain 19 vaccine suspended in saline (group A) were compared with those obtained from administration of ether-killed vaccine combined with the adjuvant (group B). In group A, the mean agglutinin titer reached its peak two weeks after vaccination then it declined sharply. In group B, on the other hand, the titers increased gradually. They remained at their height for some time, after which the agglutinin content of the serums began to decrease. Thus, as judged by the trend of the agglutinin titers, emulsification of the killed vaccine in falba and mineral oil appeared to retard the absorption of the inoculated antigen. An additional dose of killed Brucella with adjuvant administered six months after the initial injection of the vaccine was followed by a sharp rise in agglutinin production. Recently, Larsen[12] studied the immunizing quality of an ether extract of *Br. abortus* emulsified in mineral oil with falba. The guinea pigs were challenged five or six weeks after immunization, and he found that addition of killed tubercle bacilli was necessary to increase the antigenicity of the product.

The present immunization studies were carried out under field conditions, as well as in a limited challenge experiment, and the observations reported here extend over the first gestation period. The herd was divided into three groups at random, the only consideration being given to the pregnancy status of the animals, so that an equal number in group A and in group B were beyond the fourth month of gestation at the time of vaccination. Eight animals in group A aborted, 6 of which had been vaccinated in their fifth or sixth month of pregnancy. In addition, 1 animal vaccinated in the sixth month of pregnancy calved prematurely, and the calf died within twenty-four hours. Altogether, 3 fetuses aborted by animals in group A yielded a CO_2-sensitive strain of *Br. abortus*. According to the time intervals between vaccination and abortion, these animals could possibly have been in the incubation stage of the disease at the time of vaccination, or they may have become infected soon after vaccination. In another case of abortion, an aërobic strain of *Br. abortus* which had the characteristics of strain 19 was isolated. The cause of the other four abortions in group A could not be determined, but in one of these there was a possibility of Brucella infection, as judged by a rise in the agglutinin titer after the abortion. A similar increase in the agglutinin concentration was noted after the premature parturition mentioned previously, in which case the calf was born weak and died the next day. Only 16 of 26 animals in group A produced normal calves, and 1 animal has not freshened yet.

Of 25 animals in group B, 22 calved normally, 2 animals have not yet freshened, and 1 aborted. A CO_2-sensitive strain of *Br. abortus* was isolated from the fetus and, according to the interval between vaccination and abortion, it may be assumed

that the animal had become infected before or shortly after vaccination. Since there was no abortion in this group attributable to the injection of ether-killed Brucella, it would appear that there is no danger from injecting multiple doses of the killed vaccine into cattle even in an advanced stage of gestation. Thus, if such a product were found efficacious in producing resistance against brucellosis in cattle, it would constitute a valuable immunizing substance which could be used regardless of age or stage of pregnancy. Furthermore, such a product could prove valuable in the revaccination of adult animals previously vaccinated as calves.

In group C, which was the smallest of the three groups, 1 animal aborted a pair of twins, but no evidence of Brucella infection could be found.

Studies of the serologic reactions as well as of the breeding and parturition data in this herd will be continued, to obtain information which may throw further light on the biologic activity of ether-killed Brucella with adjuvant.

In the challenge experiment, the 2 heifers which had been injected with ether-killed vaccine and the 2 heifers which had received strain 19 vaccine withstood the artificial exposure to virulent *Br. abortus*. None of these 4 animals showed any sign of infection before or after parturition. On the other hand, 1 of 2 controls became definitely infected, as manifested by the isolation of *Br. abortus* from the aborted fetus. This animal also showed a marked rise in body temperature about two weeks before abortion, which may have been the result of a systemic reaction to the infection. The other control animal remained negative to the agglutination test, calved normally, and no Brucella was isolated from the placenta, colostrum, or milk. This was the only animal which at the time of challenge showed evidence of loss of Brucella suspension instilled into the conjunctival sac. Thus, it would appear that this heifer was not exposed to the same challenge dose as the other 5 animals. It is intended to repeat a similar experiment with a larger number of animals, which should lead to more conclusive results.

SUMMARY

1) In a herd in which brucellosis had existed for about a year, 73 cattle negative to the agglutination test and in different stages of the reproductive cycle, ranging from nonpregnant status to seven months of pregnancy, were divided into three groups which were treated as follows: group A was injected with strain 19 vaccine; group B was injected with ether-killed *Brucella abortus* combined with falba and mineral oil; and group C was left as controls. Two heifers from each group were used in a challenge experiment, which left 67 animals in the herd studied under field conditions.

2) Sixty-three of the 67 animals have completed the first gestation period after vaccination with the following results:

a) Of 26 animals in group A, 8 aborted, 1 cow calved prematurely and the calf died, 16 animals produced normal calves, and 1 animal has not freshened yet. Three of the aborted fetuses yielded a CO_2-sensitive strain of *Br. abortus* on cultures. In a fourth case, an aërobic strain of *Br. abortus*, possessing the characteristics of strain 19, was isolated. The cause of the other four abortions in this group could not be definitely determined.

b) Of 25 animals in group B, 1 animal aborted, 22 calved normally, and 2 animals have not freshened yet. A CO_2-sensitive strain of *Br. abortus* was isolated from the aborted fetus.

c) Of 16 animals in group C, 1 cow aborted a pair of twins, but there was no evidence of Brucella infection.

As judged by the intervals between vaccination and abortion, the 4 animals of groups A and B, from whose fetuses a field strain of *Br. abortus* was isolated, may have become infected shortly before or after vaccination.

3) In a challenge experiment, 2 heifers injected with ether-killed vaccine combined with falba and mineral oil and 2 heifers injected with strain 19 vaccine withstood a challenge dose of 166×10^4 *Br. abortus* which produced a typical infection in 1 of 2 control heifers. (The other control animal showed evidence of loss of Brucella suspension from the conjunctival sac at the time of challenge.) All of the 6 heifers were in the fifth month of gestation at the time of challenge.

4) No ill effects were noticed from injections of the saline-in-oil emulsion of ether-killed *Br. abortus* into cattle in dif-

ferent stages of gestation. On the other hand, it is not considered advisable to administer strain 19 vaccine to animals in an advanced stage of pregnancy. In this study, at least 1 case of abortion was attributable to vaccination with strain 19 vaccine beyond the fourth month of pregnancy.

5) The agglutinin titers in the cattle injected with strain 19 vaccine were highest soon after vaccination; then they declined sharply. On the other hand, injections of ether-killed *Br. abortus* combined with falba and mineral oil stimulated agglutinin production which reached the peak for the group two months after administration of the vaccine. The titers then remained quite constant for two months, after which they showed a decline. However, administration of another dose of the ether-killed vaccine six months after the original vaccination was followed by a marked rise in agglutinin production.

6) Further studies are needed to determine the antigenicity and the immunizing quality of ether-killed Brucella with adjuvants in cattle.

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Studies of Electrolytes in Body Fluids of Dairy Cattle
I. Effects of Water Diuresis on Renal Excretion of Several Elements

A. F. SELLERS, V.M.D., M.Sc., Ph.D., and M. H. ROEPKE, Ph.D.

St. Paul, Minnesota

AN EFFORT was made to learn something of the normal rates of renal excretion of several electrolytes in cows and whether those rates were affected by the rate of diuresis.

METHODS

Four nonlactating adult Holstein-Friesian cattle in late pregnancy (last 2 to 4 weeks), 4 in midlactation (4 to 5 months), 3 of which were one to three months pregnant, and 1 nonpregnant animal (No. 633) were used. These animals were continuously confined in box stalls for several days preceding study. This procedure was adopted with a view toward standardizing environmental conditions and so that feed consumption could be observed and recorded. One nonpregnant heifer was similarly treated.

Water diuresis was induced by slowly (15 min.) introducing approximately 10 gal. of lukewarm tap water through a stomach tube into the forestomachs. This was preceded by a control quantitative collection of urine. This urine was collected over a two-hour period following emptying the bladder by induced urination and/or catheterization. At the end of the two-hour control period, the bladder was emptied, and the urine obtained was added to any urine passed voluntarily during the control period. Water was then administered as described. The preinjection sample was compared with a total collection over the two-hour period during and following water administration.

The volume of the urine specimens was measured, and the specific gravity at 25 C. determined. A clinical urinometer standardized pyknometrically was used.

Urine specimens were routinely preserved by the addition of concentrated nitric acid and a few drops of toluene. Five to 10 ml. of acid added to approximately 500 ml. of urine were usually sufficient to prevent carbonate precipitation and growth of microörganisms on standing.

Calcium was determined by the method of Wang.[16] Magnesium was determined by a modification of the titan yellow method of Kolthoff,[9] as described by Pieters *et al.*[15] Chloride was determined by the Volhard-Harvey method.[5] Total phosphate was determined by the method of Pincus and Malot, as described by Peters and Van Slyke.[14]

Sodium and potassium determinations were made flame photometrically, using the internal standard procedure.[1,4,8,12] Urine dilutions of 1 : 100 to 1 : 250 were usually required for the urine potassium, and 45 : 50 to 1 : 50 dilutions for the urine sodium determinations. Interfering elements[11] were not added to urine sodium and potassium standards, due to the wide variability of levels observed. Further, it was noted that with solutions of sodium, calcium, and magnesium salts, prepared to represent maximal interferences so far reported in bovine urine, interference effects in so far as sodium and potassium determinations were concerned were within the error of the respective methods. The per cent error due to potassium interference became considerable only at extremely low sodium concentrations. No appreciable error was noted by Berry *et al.*[4] in sodium and potassium determinations by the internal standard flame photometric procedure, with nitric acid concentrations up to and including 0.8 M. Additions of nitric acid to urines in the present series could in no case have resulted in a concentration of nitrate greater than 0.4 M.

Levels of all urine constituents determined were calculated and recorded as milliequivalents per liter of urine.

RESULTS

Table 1 summarizes the results on 4 animals in midlactation, 1 nonpregnant heifer, and 4 animals in late pregnancy. No marked change was noted in the feed consumption of these animals prior to the respective experiments.

The absolute excretion of a given element, on an average basis per hour, is given by CV, where C and V are the concentration and average volume of urine per hour, respectively. The change in average

From the Division of Veterinary Medicine, University of Minnesota, St. Paul.

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excretion per hour with diuresis is represented as $\triangle E$ (table 1): $C_2 V_2/C_1 V_1 = \triangle E$.

Table 1 shows differences in absolute excretion rates of the substances studied under these conditions. It would appear that the concentrations of magnesium and of potassium were inversely proportional to the volume. From this, it follows that the change in the average absolute excretion per hour of these elements should be about unity and that the Mg: K ratio should remain reasonably constant before and during diuresis. It will be noted in table 1 that the average change in absolute excretion with diuresis was 1.2 in the cases of magnesium and potassium. In table 2, showing absolute excretions of the several elements calculated as ratios before and during diuresis, comparison of prediuresis with diuresis values of the Mg: K ratio appeared to show fair agreement of these values, in marked contrast to ratios calculated for other pairs of elements.

The observed relationship between the urine magnesium and potassium concentrations to the volume change with diuresis appeared to hold as well in the animals in late pregnancy as in those in midlactation. Further, the relationship appeared to hold whether the volume change was as low as threefold or as high as sevenfold, in terms of average volume of urine excreted per hour.

In contrast, the other elements studied appeared to be characterized by "washing out," *i.e.*, the average absolute excretion per hour being proportional to the volume. Thus, in the cases of calcium, sodium, chloride, and total phosphate, the mean change in average absolute excretion per hour with diuresis was considerably greater than unity, being increased severalfold (table 1). It will be noted from the table that the $\triangle E$ values for calcium and sodium show a wide spread owing to 1 individual in each instance. The single disparate value was not included in calculation of the average changes in excretion for calcium and sodium shown in table 1, so as to give a truer picture of the excretion of these elements.

It will be noted from table 2 that both the absolute Ca: Mg and Ca: K ratios appear consistently to be raised. This indicates that calcium tended to be washed

TABLE I—Change in Absolute Renal Excretion of Several Elements with Water Diuresis

Animal (No.)	Status	Sp.g.*	Ave. vol./hr., (liters)	Calcium			Magnesium		Sodium		Potassium		Chloride		Total phosphate	
				Conc., mEq./l.	ΔE**	Conc., mEq./l.	ΔE	Conc., mEq./l.	ΔE	Conc., mEq./l.	ΔE	Conc., mEq./l.	ΔE	Conc., mEq./l.	ΔE
781	Midlact.	1.012	1.30	0.83		18.0		0.18		134		14.0		6.3	
		1.002	5.00	0.48	2.2	4.2	0.9	0.28	5.90	43	1.20	11.0	2.90	5.3	3.2
633	Midlact.	1.021	0.54	1.40		18.0		31.00		203		3.6		9.5	
		1.008	1.80	1.10	2.6	6.7	1.2	7.80	0.83	70	1.10	3.6	3.30	6.8	2.4
658	Midlact.	1.020	1.10	1.50		23.0		89.00		128		5.5		7.4	
		1.003	3.90	0.73	1.7	10.0	1.5	27.00	1.10	37	1.00	6.9	4.40	8.4	3.9
756	Midlact.	1.035	0.64	0.39		24.0		91.00		302		29.0		. .	
		1.006	3.70	0.28	4.2	5.1	1.2	47.00	3.00	51	0.98	11.0	2.20	. .	. .
751	Nonpreg. heifer	1.008	0.76	0.19		13.0		0.28		93		8.6		7.4	
		1.001	5.10	0.59	21.0a	3.3	1.7	0.37	8.80	23	1.70	. .	0.78	6.3	5.6
707	Late preg.	1.007	0.84	0.23		13.0		0.43		74		4.5		6.3	
		1.001	2.70	0.15	2.1	3.3	0.8	4.70	35.00a	26	1.10	7.0	5.00	6.3	3.2
634	Late preg.	1.034	0.25	3.30		81.0		34.00		223		50.0		5.9	
		1.009	0.90	4.20	4.5	25.0	1.1	14.00	1.50	67	1.10	6.3	0.46	5.9	3.6
574	Late preg.	1.008	1.30	0.75		14.0		2.30		95		5.1		6.3	
		1.001	4.00	0.70	2.7	5.0	1.1	7.10	6.40	42	1.30	. . .	0.59	8.4	3.8
659	Late preg.	1.040	0.48	7.70		46.0		6.50		361		16.0		9.5	
		1.004	3.30	2.70	2.4	9.6	1.4	3.90	4.00	57	1.10	4.5	1.90	5.3	3.8
				Ave.	2.8		1.2		3.9		1.2		2.4		3.4

*Upper line, prediuresis; lower line, during diuresis. **$C_2 V_2/C_1 V_1 = \triangle E$, change in rate of absolute excretion. aSingle disparate value excluded from computation of the average.

TABLE 2—Change in Ratios of Absolute Renal Excretion of Several Elements with Water Diuresis

Animal (No.)	Ca : Mg*	Ca : Na	Ca : K	Ca : Cl	Ca : P	Mg : Na	Mg : K	Mg : Cl	Mg : P	K : Na	K : Cl	K : P	Na : Cl	Na : P	Cl : P
781	0.05	4.800	0.006	0.06	0.13	100.00	0.13	1.30	2.80	742.0	9.7	21.0	0.01	0.03	2.20
	0.11	1.700	0.011	0.04	0.09	15.00	0.16	0.38	0.78	155.0	3.9	7.9	0.03	0.05	2.00
633	0.08	0.050	0.007	0.40	0.15	0.59	0.09	5.30	2.00	6.5	58.0	22.0	8.90	3.30	0.37
	0.17	0.140	0.016	0.31	0.17	0.86	0.10	1.90	1.00	9.0	19.0	11.0	2.20	1.20	0.54
658	0.07	0.020	0.012	0.28	0.21	0.26	0.18	4.10	3.00	1.4	23.0	17.0	16.00	12.00	0.74
	0.07	0.030	0.019	0.10	0.09	0.37	0.27	1.40	1.20	1.4	5.3	4.4	3.90	3.20	0.82
756	0.02	0.004	0.001	0.01	...	0.26	0.08	0.83	....	3.3	10.0	...	3.10	....	...
	0.06	0.006	0.005	0.03	...	0.11	0.09	0.46	....	1.2	5.0	...	4.30	....	...
751	0.01	0.670	0.002	0.02	0.02	48.00	0.14	1.50	1.70	334.0	11.0	12.0	0.03	0.04	1.10
	0.18	1.600	0.026	...	0.09	8.90	0.15	...	0.53	67.0	...	3.7	....	0.06	...
707	0.02	0.530	0.003	0.05	0.04	31.00	0.18	2.90	2.10	172.0	16.0	12.0	0.10	0.07	0.72
	0.05	0.030	0.006	0.02	0.02	0.69	0.13	0.47	0.53	5.6	2.7	4.1	0.68	0.76	1.10
634	0.04	0.100	0.015	0.06	0.55	2.40	0.36	1.50	13.00	6.5	4.3	37.0	0.65	5.70	8.70
	0.17	0.290	0.063	0.67	0.72	17.00	0.37	3.90	4.20	4.8	11.0	11.0	2.30	2.50	1.10
574	0.05	0.230	0.008	0.15	0.12	4.20	0.15	2.70	2.20	29.0	19.0	15.0	0.65	0.52	0.80
	0.14	0.100	0.017	...	0.08	0.71	0.12	...	0.59	5.6	...	4.9	...	0.82	...
659	0.17	1.200	0.021	0.48	0.81	7.10	0.13	2.90	4.80	55.0	22.0	38.0	4.00	0.67	1.70
	0.28	0.680	0.047	0.59	0.49	2.50	0.17	2.10	1.80	15.0	13.0	10.0	0.87	0.72	0.83

*Upper line, prediuresis; lower line, during diuresis.

out by water diuresis. As discussed above, the absolute Mg : K ratio tended to remain the same, no matter what the volume change. The absolute Mg : P and K : P ratios both seemed regularly to be lowered by water diuresis; phosphate was thus washed out. The remaining ratios appeared irregular from animal to animal.

DISCUSSION

The apparent constancy of absolute magnesium and potassium excretion despite approximately three- to sevenfold changes in urine volume would seem to indicate first, that these two substances are handled in like manner by the bovine kidney. Secondly, it would appear that consideration might be given to the use of either of these elements to measure the rate of glomerular filtration in this species if it were found that their excretion rates increase over wide limits in simple, direct proportion to the plasma concentration (Smith[16]).

Wolf[20] found that potassium behaves in many experiments of short duration in normal man as a no-threshold substance. It is believed, however, by this author, actually to have a low but undetermined threshold of retention. Tarail and Elkinton[17] found that the capacity of the kidney to conserve potassium is low in human beings; in both normal and abnormal subjects, potassium excretion persisted when the potassium intake was negligible. Tubular secretion of potassium is demonstrable when potassium salts are given, in dogs[2, 10] and in human beings.[3]

The absolute excretions of calcium, sodium, chloride, and total phosphate seem to be increased by water diuresis in the bovine species. This raises the question of the possible effects of a sudden change to lush pasturage on balances between elements which seem critical in regulation of function in the animal body, such as the potassium : calcium relationship.

In man, water diuresis tends to carry considerable quantities of both sodium and potassium out of the body,[16] as well as chloride.[13] The normal excretion of phosphate is not increased in water diuresis in human beings.[7, 8, 19]

SUMMARY

1) Magnesium and potassium would appear to be excreted by the same mechanism, in the bovine kidney. The absolute excretion of each, at least in experiments of short duration, seems independent of the volume of urine.

2) Calcium, sodium, chloride, and total phosphate appear to be excreted in larger amounts with water diuresis than before such diuresis is imposed.

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Research on Foot-and-Mouth Disease. III. The Cultivation of the Virus on a Practical Scale in Explantations of Bovine Tongue Epithelium

H. S. FRENKEL

Amsterdam, Holland

AFTER HAVING cultivated the virus of foot-and-mouth disease as described in a previous paper,[1] the problem was to find a practical method for large-scale cultivation of this virus for use in vaccine production. Using the same principles described previously, experiments were conducted using stainless steel containers of 500-ml. capacity for the cultures instead of Erlenmeyer flasks.

MATERIALS AND METHODS

In these trials, the first difficulty to be overcome was the proper control of aëration. The outlet tube of the steel container was connected with a bottle half filled with water through which the gases were bubbled. Virus so cultivated was found to be comparable with that propagated in Erlenmeyer flasks.

After having determined that the small stainless steel containers were satisfactory for cultivation of the virus, larger tank containers were devised in which epithelium from 40 to 60 adult bovine tongues could be handled. A tank (fig. 1) of 60-liter capacity was fitted with a closely fitting cover that might be tightly closed by means of screw clamps and a rubber gasket. The cover had two glass windows making it possible to look into the container with the aid of illumination from an electric bulb provided at one window.

For aëration, a T-shaped, stainless steel tube with small holes at the ends was fitted in the cover, and an electric motor was mounted on one of the handles of the tank to rotate the tube slowly. Later, this system of aëration and stirring was changed to one in which two 5 L 3 Chamberland* filter candles, 1.5 cm. in diameter and 15 cm. in length, were affixed to the aëration tube at a level as near to the bottom of the tank as possible (fig. 2). The apparatus, which is known as Mag-Mix, might be used for stirring the culture. With this arrangement, a sterilized magnetic piece of iron

Fig. 1—Cultivation tank, showing clamped lid with windows, aëration and stirring mechanism, and air outlet.

sealed in glass or plastic may be placed in the bottom of the tank. The Mag-Mix, an electric motor with a strong magnet affixed, is placed directly under the tank, thus rotating the iron inside the tank. While sterile conditions may be maintained by this means, the process is not very dependable.

Using the large stainless steel tanks in an incubator room and the same general techniques previously described,[1] we succeeded in cultivating virus on a large scale with a 50 per cent end point infective dose in dilutions of $3x10^{-6}$ to $3x10^{-6}$.

After a satisfactory container for large-scale cultures had been developed, the next problem

From the State Veterinary Research Institute, Amsterdam, Holland. The author is director of the Institute.

The first paper of this series, "Histologic Changes in Explanted Bovine Epithelial Tongue Tissue Infected with the Virus of Foot-and-Mouth Disease," appeared in the American Journal of Veterinary Research, vol. x, No. 35, April, 1949, pp. 142-145.

The second paper of this series, "Research on Foot-and-Mouth Disease. II. The Cultivation of the Virus in Explantations of Tongue Epithelium of Bovine Animals," appeared in the American Journal of Veterinary Research, vol. xi, No. 41, October, 1950, pp. 371-373.

*These correspond, in porosity, to those designated L 3 in the United States.

was to devise more practical means of collecting and mincing the lingual epithelium. Using the techniques formerly described,[1] a good worker can handle about four tongues per hour.

Fig. 2—Cultivation tank opened to show arrangement of Chamberland filter candles.

Collection of epithelium from 100 tongues would take about twenty-five man hours or four good workers might do the job in about six hours.

To lessen the time and tedium of collection of the epithelium by hand, we conceived of the utilization of a Berkel slicing machine (Model P 14), such as is commonly used for slicing fish. This machine has a rapidly rotating circular blade which is operated by an electric motor. The disc-shaped knife of the machine is washed with 70 per cent alcohol, and during the slicing operation it is kept moist by continual dripping of sterile Tyrode's solution to facilitate cutting.

In order to stretch and fasten the tongue firmly to facilitate manipulation of its epithelial surface over the edge of the rapidly rotating blade of the slicing machine, we adapted the metal lid of an old-fashioned 40-liter milk can (fig. 3). This had just the right circumference to accommodate the average-sized tongue, after removal of the pharynx and larynx. A wedged-shaped opening is made in one side of the cylindric lid, through which the base or bulb of the tongue is introduced from the outside and firmly fastened in the tapering opening. On the opposite side of the lid, a rectangular opening is provided. The tongue is wrapped around the outer surface of the cylinder, dorsal surface outward, and the ventral surface of the tip of the organ is affixed on hooks on the inside of a curved copper plate which laps over the rectangular slot. The tongue may then be stretched tightly around the cylinder by means of traction on a heavy cord attached to the plate covering the rectangular slot, the cord being wrapped around a small knob beyond the side opening to provide the desired tension. The hooks to which the tip of the tongue are attached are on the inside of the movable copper plate to eliminate possible contact with the cutting edge of the meat slicer.

With the tongue fastened in a curve around the perimeter of the metal cylinder, it is thoroughly cleansed with lukewarm water and a brush. After this, the organ is swabbed with alcohol (70%) and allowed to stand for some minutes to let the alcohol evaporate.

With a rotary manipulation, an operator then applies the outer surface of the mucous surface of the tongue parallel or slightly tangent to the rapidly rotating cutting disc of the meat slicer. The superficial portion of the mucosa is cut off and discarded. Several sheets are removed to a depth where the bluish submucous tissue becomes visible. As the operator continues to remove the epithelial sheets, a helper grasps succeeding slices with sterile forceps as they are cut off and transfers them to a sterile cylindric glass container of about 1-liter capacity. The epithelium from four or five adult tongues is placed in each container with 250 ml. of the modified Baker's solution used for the culture.[1]

Fig. 3—Metal cylinder around which tongue is fixed for removal of epithelium.

Only about two minutes are required by an experienced, skilful operator to remove the epithelial tissue from one tongue. As many as 35 to 40 tongues may be so handled in an hour. It sometimes happens that some of the muscle tissue is included in the slices removed from the tongues. If too much of this is included in the material for the culture, the formation of lactic acid in the tissue becomes harmful to the virus, but it has been found

that minute quantities of muscle are not noxious.

As discussed in our previous paper,[1] the outer layers of the mucosa were at one time discarded, chiefly because of difficulties to be expected from bacterial contamination, and only the lower layers were incorporated in the culture. The work of our co-worker, Dr. Howard Dunne,** suggested the practical utilization of the whole lingual mucous membrane. This was made more practical by the use of penicillin and streptomycin as antibiotics, as previously referred to. Results to date indicate that the titer of virus propagated on the whole epithelium equals or may even exceed that of virus produced on the deeper layers alone.

Until recently, the mincing of the epithelium for the culture was done by hand, using scissors to divide the tissue contained in Petri dishes protected by covers during the operation. This is a very tedious and tiring task, requiring from twelve to fifteen minutes to prepare the epithelium from one tongue. The apparatus† illustrated in fig. 4 was designed by W. de Soet and J. Lokker, the Institute's instrument makers, for mechanically dividing the epithelial tissue.

When using a pair of scissors for shearing material into fine bits, one usually operates with one finger holding one blade practically fixed, the second moving the other blade. This principle was applied in devising equipment for mincing the tissue by means of a series of mechanically operated shears or clippers. The shears are made in two parts: 1) a Y-shaped flat portion that is fixed to a backboard, and 2) a moving blade which slides over the first and is moved from side to side by means of an attachment with the ball bearing supported, double track, or guide from a steel filing cabinet. One portion of the double track is fixed on a backboard and the other, to which the movable part of the scissors is attached, is free to move lengthwise within a channel. The inner part of this assembly is moved by an eccentric connection with the shaft of an electric motor.

The glass containers of tissue and fluid are mounted on eccentrically arranged, rotatable holders where they are held in place with a ring of elastic rubber. The containers, which are inclined considerably to lessen air contamination and, in addition, are covered by a plastic cap, are operated by the same motor which moves the scissors. The scissors are easily removable for sterilization. With this equipment, the tissue may be uniformly clipped in a relatively short time. Using two containers and two scissors, the tissue from ten tongues may be processed within ten minutes.

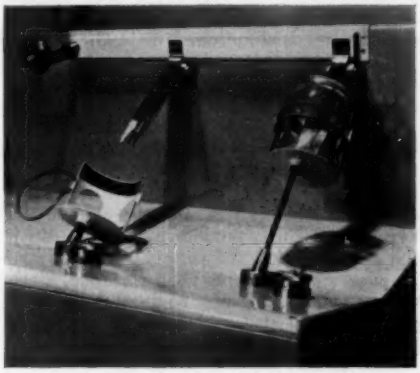

Fig. 4—Clipping machine.

With all ingredients of the culture ready, they are quickly drawn into the sterilized tank by vacuum, and incubation may proceed immediately. It has been our experience that the penicillin and streptomycin used have not always retarded bacterial contamination, which may seriously reduce the virus titer. We have found chloromycetin to be useful in controlling such contamination when 250 mg. of the substance are added to each ten tongues being used.

DISCUSSION

Development of a wholly practical system of mass culture of foot-and-mouth disease virus had many advantages over the propagation of the virus in living cattle, for preparation of vaccine. For animal propagation of the virus, large numbers of cattle are required in order to produce the quantities necessary for vaccine production. The infection of 500 animals weekly, as has been necessary in the Netherlands, has required separate, spacious isolation quarters for each type of the virus being so propagated. The slaughter of the infected animals twenty-four hours after inoculation demands an isolated abattoir and many workmen. The carcasses of the slaughtered animals must be handled in accordance with the requirements of the laws of the country. When the meat is to be consumed, large, refrigerated storage rooms are essen-

**This portion of the work was carried out by Howard W. Dunne as a part of the coöperative program of research on foot-and-mouth disease between the State Veterinary Research Institute, Amsterdam, and the Bureau of Animal Industry, Washington, D. C.

†The equipment described in this paper was constructed in entirety in our Institute by W. de Soet in collaboration with J. Lokker. They succeeded admirably in developing the rough ideas given to them by the writer.

tial for keeping the carcasses for a sufficient time to allow formation of adequate quantities of the virucidal lactic acid in the meat. Because of these factors, the costs of vaccine production with virus from inoculated cattle are very high, even though the meat from these animals is salvaged for human consumption. The quality of the carcass is materially affected by the infection. The suffering of the inoculated animals is also a consideration.

On the other hand, *in vitro* cultivation of the virus eliminates or materially decreases most of these objections. Most of the costly installations and attendant precautions required when the virus is propagated in the living animal may be done away with. With the described culture procedures, even the tongues may be safely used for food, since they have not become contaminated with the virus and the only alteration to them is that the mucosa has been removed.

In our opinion, this method of virus production can be used in almost every country, chiefly because it does not require expensive installations. The greatest expense would be in the relatively small isolation boxes and stables necessary for innocuity and potency tests of the vaccine.

SUMMARY

A description is given of procedures and facilities for mass production of foot-and-mouth disease virus to be used for preparation of preventive vaccine.

Chloromycetin was useful in controlling bacterial contamination.

The advantages of the cultured virus over that generally used for vaccine production are discussed.

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Fertility and Bacterial Content of Diluted Bull Semen Treated with Various Concentrations of Dihydrostreptomycin Sulfate

H. L. EASTERBROOKS, D.V.M.; P. HELLER; M. LIEBERMAN, M.S.;
W. N. PLASTRIDGE, Ph.D.; E. L. JUNGHERR, VET. DIPL., D.M.V.

Storrs, Connecticut

THE ADDITION of streptomycin and/or penicillin improves the fertility of diluted bull semen under certain conditions.[1,2,4,6,7,11] Streptomycin has value when used in a routine breeding operation.[6,7] Fertility improvement was as great when streptomycin alone was added to semen diluted in egg yolk-citrate-sulfanilamide as when streptomycin and penicillin were added in combination.[10]

Hennaux *et al.*[12] reported increased motility over nontreated controls in diluted semen stored nine days following treatment with 3 mg. of streptomycin per milliliter. Almquist *et al.*[3] from *in vitro* studies reported 1,000 μg. per milliliter of streptomycin to be nontoxic to spermatozoa as well as inhibitory to a high percentage of bacterial contaminants. This level has been used and fertility increased thereby.[2,4] Comparable fertility increases resulted from the addition of 100 μg. of streptomycin.[6,7]

It was the purpose of this study to test various levels of dihydrostreptomycin sulfate[8,9] to determine their comparative values for improving the fertility of diluted semen and to measure their relative effectiveness in controlling contaminating microorganisms.

MATERIALS AND METHODS

Four Holstein-Friesian and 4 Guernsey bulls owned by the Connecticut Artificial Breeding Association, and at the time in routine use, were assigned to the experiment. Semen was collected four times from each bull at exactly fourteen-day intervals. Following collection, the semen was diluted at the approximate rate of 1 to 75 in a diluent composed of one part egg yolk and four parts 2.9 per cent sodium citrate ($Na_3C_6H_5O_7.5\frac{1}{2}H_2O$) containing 0.6 per cent sulfanilamide. Following dilution, the semen was divided into four equal portions and dihydrostreptomycin sulfate solutions added to each to produce the desired final concentrations of the drug. The streptomycin solutions had been previously prepared, measured accurately into small glass vials, and placed in a freezer for storage up to a few minutes before they were used. The concentrations of 100 μg. per milliliter, 300 μg. per milliliter, 900 μg. per milliliter, and a no treatment control were the four levels tested. These concentrations of streptomycin were selected because they closely embraced the concentrations of streptomycin which had been reported as being effective for improving fertility.

The inseminators, 12 in number, were divided into four groups each having the potential of an equal number of cows to be bred during the course of the study. Each of the four groups received semen at each treatment level from each bull during the experimental period.

First services only were considered, and the sixty- to ninety-day nonreturns to first artificial service percentages recorded on the Association records were used as the basis for comparison.

Each day, 0.2 ml. of raw semen and 2.0 ml. each of the four treatment portions from each bull used were delivered to a city near the University of Connecticut along with the day's shipment to the local inseminator. A University-owned truck picked up the shipment and delivered it to the Department of Animal Diseases between eight and twelve hours from the time of its processing. Examinations were conducted within a half-hour after arrival of the samples. Bacterial counts were made from beef infusion-blood agar plates streaked with 0.01-ml. amounts of raw semen and the four treatment portions after serial dilution. Sam-

From the Department of Animal Diseases, Storrs Agricultural Experiment Station, University of Connecticut, Storrs.

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Mr. Heller is manager of the Connecticut Artificial Breeding Association, Woodbridge.

At present, Mr. Lieberman is at the Graduate School, University of Pennsylvania, Philadelphia.

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ples were placed in a 5 C. refrigerator and all but the raw semen reëxamined twenty-four hours later.

Type isolations were made by taking material directly from the tubes as received and streaking on blood agar. Plates were incubated aërobically. The usual methods for determining type of colony, morphology, and biologic reactions were used in identifying the cultures.

TABLE 1—Fertility of Diluted Semen Containing Various Levels of Dihydrostreptomycin Sulfate

	Micrograms of streptomycin per ml. of diluted semen			
	0	100	300	900
Holstein-Friesian bulls:				
1	35/ 53=66.0*	48/ 67=71.6	51/ 67=76.1	40/ 56=71.4
2	22/ 48=45.8	29/ 50=58.0	46/ 60=76.7	48/ 68=70.6
3	48/ 70=68.6	46/ 69=66.7	39/ 50=78.0	24/ 40=60.0
4	43/ 66=65.2	60/ 74=81.1	46/ 56=82.1	40/ 51=78.4
All Holstein-Friesians	148/237=62.4	183/260=70.4	182/233=78.1	152/215=70.7
Guernsey bulls:				
1	36/ 63=57.1	31/ 59=52.5	46/ 61=75.4	37/ 58=63.8
2	33/ 58=56.9	31/ 44=70.5	41/ 56=73.2	35/ 49=71.4
3	38/ 66=57.6	51/ 68=75.0	54/ 69=78.3	43/ 59=72.9
4	41/ 58=70.7	47/ 65=72.3	38/ 53=71.7	52/ 62=83.9
All Guernseys	148/245=60.4	160/236=67.8	179/239=74.9	167/228=73.2
All bulls	296/482=61.4	343/496=69.2	361/472=76.5	319/443=72.0

*Nonreturns to first service over total services equals nonreturns per cent.

RESULTS AND DISCUSSION

Fertility of Diluted Semen.—The overall results showed sixty- to ninety-day nonreturns percentages of 61.4, 69.2, 76.5, and 72.0 for the treatment levels of 0, 100, 300, and 900 μg. of streptomycin per milliliter, respectively, as shown in table 1.

The data, when tested by the analysis of variance,[14] revealed a highly significant ($P<0.001$) difference between levels of streptomycin and a significant ($P<0.05$) difference between individual bulls. Although sufficient data were not collected to precisely predict the optimum level for treatment of semen with streptomycin, they do suggest that neither 100 nor 900 μg. per milliliter is optimum for improving fertility but instead some concentration in between. It seems to the writers that the reason for this might be that, even though diluted semen is not visibly harmed by higher concentrations at storage temperatures,[3,12] under practical conditions in the field some decrease in fertility may result. Further, the action of streptomycin may not rest in bacterial control, alone, but may also be involved in cellular metabolism of the spermatozoa.

When a curve is graphically fitted to the response data, it indicates that the optimum response occurs above rather than below 300 μg. per milliliter. Coupling with this the fact that Plastridge and Easterbrooks[13] have found a concentration of 500 μg. of streptomycin per milliliter necessary to inhibit *Vibrio fetus* organisms in diluted bull semen held at 5 C., it seems that the 500 μg. per milliliter level is the most practical for recommendation at this time. More data to define the optimum level are needed.

Bacteriology of Streptomycin-Treated

TABLE 2—High, Low, and Average Bacterial Counts per Milliliter of Diluted Bull Semen Containing Various Levels of Streptomycin

Concentration of streptomycin per ml.	Time of examination	
	8 to 12 hours	32 to 36 hours
Raw semen untreated	Less than 100 to 390,000 Ave. 77,859	.....
Diluted semen untreated	Less than 100 to 31,000 Ave. 8,627	Less than 100 to 16,000 Ave. 4,345
100 micrograms	Less than 100 to 24,000 Ave. 4,330	Less than 100 to 21,000 Ave. 2,335
300 micrograms	Less than 100 to 3,600 Ave. 604	Less than 100 to 900 Ave. 228
900 micrograms	Less than 100 to 700 Ave. 163	Less than 100 to 400 Ave. 63

Semen.—The average bacterial counts for all samples examined are shown in table 2. In general, the bacterial count was inversely proportional to the concentration of streptomycin. Bacteria-free plates were not always produced at the 900-μg. concentration. although they did occur 14 out of 32 times at eight to twelve hours and 20 out of 32 times at thirty-two to thirty-six hours at this level. It was observed that the fertility of streptomycin-treated semen was not solely dependent upon the number of microörganisms present.

Diphtheroids and micrococci were isolated with the greatest frequency. This is in agreement with available literature concerning semen bacteriology. Hemolytic streptococci, hemolytic bacilli, members of the genus Alcaligenes, *Gaffkya tetragena*, *Pseudomonas aeruginosa*, and various molds and fungi were also isolated. *Escherichia coli* was not isolated. Dondero[5] stated that *Ps. aeruginosa* was the only organism which he isolated exclusively from raw semen; all other types were also found occasionally in the unused diluents which he examined. All types isolated by the writers were found, though in far fewer numbers, in some of the samples having the highest streptomycin concentration. Proportionally more molds and fungi were isolated at the higher than at the lower or no streptomycin levels.

No correlation between the fertility of diluted bull semen and the presence of specific types of bacteria was observed.

SUMMARY

Nonreturns to first artificial service percentages were 61.4, 69.2, 76.5, and 72.0 for diluted bull semen containing 0, 100, 300, and 900 μg., respectively, of dihydrostreptomycin sulfate. Analysis of variance revealed a highly significant difference between levels of streptomycin.

Bacterial control increased with the concentration of streptomycin, however, fertility was highest at the 300-μg. level of treatment.

No correlation between fertility and any specific type of organism isolated was observed.

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Listeriosis—Field and Laboratory Studies, and Aureomycin Activity

A. ZINK, M.D.; G. C. deMELLO, A.B.; R. L. BURKHART, V.M.D.

Pearl River, New York

LISTERIOSIS presents a difficult problem from the standpoint of diagnosis and therapy. This report is concerned with bacteriologic studies on *Listeria monocytogenes* and chemotherapy of experimental infections in laboratory animals.

LITERATURE CITED

Since Murray, Webb, and Swann[1] first reported the isolation of *L. monocytogenes* from spontaneous infections of laboratory animals, in 1926, a continuously increasing number of cases has been reported. Nyfeldt[2, 2a] was the first to report isolation of the organism from man, and later Burn[3] described human cases of meningitis due to Listeria. Cases of human listeriosis have been reported from California, Massachusetts, Norway, Sweden, and South America. Some evidence that Listeria may be responsible for certain syndromes diagnosed as infectious mononucleosis (glandular fever) has been offered by Stanley.[4] The organism was established as the causative agent of "circling disease" in sheep and cattle, and has been isolated from pigs, goats, foxes, ferrets, rabbits, chinchillas, lemming, chickens, and turkeys. Graham, Hester, and Levine[5] identified Listeria as a cause of abortion in cattle, and Paterson[6] isolated it from aborted ovine fetuses. The symptomatology of listeriosis in the lower animals is so variable that the disease may be confused with rabies, parturient paresis, ketosis, brain abscess, meningitis, encephalitis, facial paralysis due to trauma, and others. A recent paper by Morris and Norman[7] suggests that the organism may also be harbored by apparently healthy animals. The literature on Listeria infections has been reviewed by Graham, Levine, and Morrill[8] and by Julianelle.[9] Listeriosis appears to be of worldwide distribution and apparently is increasing in frequency. It has been predicted that the disease may someday assume the importance of brucellosis as a public health problem.[9]

FIELD OUTBREAK

This report is concerned with the appearance of listeriosis on a New Jersey dairy farm where the history of the outbreak extended over a period of eight months. There were 22 head of cattle on this farm.

In August, 1949, 2 cows, out of the herd of 22, became acutely ill with an apparent unilateral paralysis of the ear, with anorexia, marked reduction in milk production, incoördination, and prolonged recumbency. The symptoms persisted for approximately two weeks and both animals spontaneously recovered.

In December, 1949, 2 other cows exhibited similar symptoms and were treated with sulfanilamide and a single intramuscular injection of 2 million units of penicillin in oil. Both animals recovered within two to three weeks.

On March 1, 1950, a fifth cow displayed the above symptoms, and therapy consisted of sulfanilamide administered orally and a single injection of 2 million units of penicillin in oil given intramuscularly. There was no clinical response and the animal died nine days later.

On March 3, a sixth animal exhibited a "dropped" ear and the symptoms described above. In addition, there was an elevated temperature, 104.2 F., and excessive salivation. When observed seven days later, the animal was recumbent with cold extremities and efforts to make her stand were unsuccessful. Although the animal appeared to be moribund, it was decided to put her on a course of aureomycin therapy. A single daily dose of 5 Gm. of the antibiotic was administered intravenously on each of three consecutive days, but the animal died on March 13. Santi and Serembe,[10] in studies on absorption, distribution, and elimination of aureomycin, reported that there was a concentration of 10 μg. per gram of brain five minutes following intravenous administration of 40 mg. per kilogram of body weight to rabbits or guinea pigs while, after thirty minutes, a concentration of only 1 μg. was present and, after one hour, no trace of the drug could be demonstrated in the brain. From their

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findings, it would suggest that one daily dose administered to an animal seven days after the onset of the disease, with probable central nervous system involvement, could not be expected to maintain a therapeutic concentration at the site of infection. A blood sample was taken for bacteriologic examination.

TABLE 1

	Microgram/milliliter inhibiting growth	Bacteriostatic ratio
Aureomycin HCl	0.04	1
Terramycin HCl	2.50	62
Penicillin G	10.00 (16.6 units)	250
Chloromycetin	20.00	500
Streptomycin	160.00	4,000

Since it was not possible to perform a complete autopsy, only the brain was exposed. The subdural space was distended with fluid, the blood vessels were engorged, and the cerebrum was edematous. The cerebrum was removed for bacteriologic examination.

BACTERIOLOGIC EXAMINATION

Culture Isolation.—No growth was obtained from the blood specimen.

The brain tissue was ground in a Tenbroeck grinder and plated on 5 per cent sheep blood agar. Tubes of nutrient and thioglycollate broth were also inoculated. No growth was obtained either on blood plates or in nutrient broth within five days, but the thioglycollate medium showed a pure, heavy growth of a small gram-positive rod. Subcultures on blood agar showed numerous, small, flat, smooth colonies of the same organism in pure culture. A small zone of *beta* hemolysis was produced on both horse blood and sheep blood agar. Growth characteristics and morphology were typical of Listeria. The following cultural and biochemical data describe and identify this strain:

The organism isolated was a short, gram-positive rod, 0.5 by 1.0μ, with rounded ends, exhibiting a tumbling type of motility in young cultures.

Acid was produced in glucose, maltose, trehalose, and salicin. Slow acid production was also noted in lactose, sucrose, and glycerol. No fermentation of arabinose, galactose, mannitol, sorbitol, inositol, starch, dextrin, inulin, or raffinose occurred.

There was no action on litmus milk nor liquefaction of gelatin. Catalase was produced. Methyl red reaction was positive. There was no formation of acetylmethylcarbinol, indol, or hydrogen sulfide.

Gray[11] has reported that refrigeration of bovine brain tissue suspension for five weeks to three months before culturing yields a higher percentage of recoveries of Listeria. It appears possible that bovine brain tissue may contain a growth-inhibiting factor which is sensitive to lower temperatures. Gray's technique presents the interesting possibility that Listeria infection in cattle may be more frequent than the results with ordinary cultural methods would indicate.

In view of the negative results with our primary blood-agar cultures, we stored portions of brain tissue in glycerol at 4 C. and on dry ice for three months. Subcultures to thioglycollate broth and blood agar were all negative. A suspension of the frozen tissue was prepared and stored at 4 C. for seven days. This material, which had previously been negative on culture, showed 156 colonies when 0.1 ml. was plated.

TABLE 2

Group	Treatment	Survival rate	Survival (ave. hr.)
A	Aureomycin, 2 doses of 10 mg./kg.	1/4	151
B	Aureomycin, 2 doses of 2.5 mg./kg.	0/4	70
C	Controls, no treatment	0/4	38

After ten days of storage, 0.1 ml. of the suspension on subculture showed colonies which were too numerous to count. Four weeks later, several hundred colonies were obtained in subcultures from 0.01 ml. of the same material. It should be stressed that, from the beginning, excellent growth was obtained from brain suspension in thioglycollate broth. For the primary isolation of Listeria, it appears that this medium is more suitable than blood agar. Possibly thioglycollate broth, or one or more of its constituents, may counteract the postulated growth-inhibiting factor of bovine brain tissue.

Agglutination Tests.—Blood samples were obtained from 2 cows which had been sick and had showed the described symptomatology. They were tested for agglutinins. One animal showed a titer of 1: 5,000 against *L. monocytogenes;* the other was negative.

Animal-Inoculation Studies.—Two hamsters were injected intracerebrally with 0.02 ml. of a twenty-hour culture. Both animals died within twenty-four hours and *L. monocytogenes* was recovered in pure culture from the brain, liver, and cornea.

Following the intracerebral injection of 0.03 ml. of a twenty-hour culture, 1 rabbit developed paralysis of the hind legs, back, and sphincter muscles within twenty-four hours, and died after four days. Pure cultures of the organism were obtained from the brain and liver. A sec-

ond rabbit which had been infected by the subcutaneous route was free of symptoms. The animal was destroyed on the eleventh day, and autopsy revealed nodular focal caseous necrosis in the liver, fibrous pericarditis and myocarditis, and an infarct with fibrotic scarring in the left ventricle.

TABLE 3

Group	Treatment	Survival rate	Survival (ave. hr.)
A	Aureomycin, 4 doses of 10 mg./kg.	6/6	..
B	Aureomycin, 4 doses of 5 mg./kg.	6/6	..
C	Penicillin G, 4 doses of 10 mg./kg.	0/6	77
D	Penicillin G, 4 doses of 5 mg./kg.	0/6	79
E	Controls, no treatment	0/6	74

A severe conjunctivitis developed in a third rabbit following supraconjunctival inoculation with one drop (ca. 30 million organisms) of an eight-hour broth culture. Listeria were continuously found in eye smears for eight days. The animal appeared healthy and was killed on the fourteenth day. Pathologic findings included a slight myocarditis and necrotic yellow foci in the liver. Liver cultures were positive for Listeria; the brain was negative. Following the ocular instillation of the same culture, 6 guinea pigs all developed a severe conjunctivitis after forty-eight hours and a progressive keratitis in three to four days.

Since chickens are reported to be susceptible to natural infection with Listeria, an attempt was made to infect 6-day-old white cross chicks. Twelve birds were inoculated intraperitoneally with 0.05 ml. of a yeast extract broth culture (ca. 300 million organisms). All appeared healthy on the eighth day after injection, and autopsy findings were negative. It is possible that experimental infection of chickens might be successfully accomplished by employing other routes of inoculation.

In Vitro Studies

Thermal Resistance Tests.—Since Listeria is generally believed to be a delicate organism with little resistance to adverse conditions, it appeared desirable to test its thermal death point. Three-hour broth cultures of 0.5 billion organisms per milliliter were placed in water baths of different temperatures. Subcultures were made with 0.5 ml. of inoculum at different intervals. Cultures held at 60 C. remained viable for ten minutes. If denser cultures of a seven-hour growth were used, growth was still obtained after five minutes at 100 C., but not after ten minutes.

Sensitivity to Antibiotics.—The use of sulfonamides, penicillin, and streptomycin has given disappointing results in listeriosis. Handelman[12] reported that penicillin was without effect in a case of human listeriosis. Describing an outbreak in sheep, Ryff and Lee[13] found that streptomycin, sulfathiazole, and sulfamerazine were of no value, while sodium sulfapyridine and penicillin appeared to be of only slight benefit. Sulfanilamide was reported to be of little value by Graham, Levine, and Morrill.[8] *In vitro* studies by Foley, Epstein, and Lee[14] showed that Listeria is highly resistant to penicillin. This was confirmed by Felsenfeld *et al.*,[15] who reported that the organism was sensitive to low levels (0.03 to 1.25 μg./ml.) of aureomycin, neomycin, and polymixin D. Gray, Thorp, and Laine[16] also reported that growth was inhibited by aureomycin in concentrations of 0.25 to 0.75 μg. They showed that this antibiotic was also very active *in vivo*. On the basis of their *in vitro* studies, Gray, Stafseth, and Thorp[17] considered streptomycin to be contraindicated, since the organism rapidly develops a fastness to this drug. Long and his associates[18] consider aureomycin to be the drug of choice at the present time for the treatment of listeriosis.

Comparative *in vitro* sensitivity tests were made by the tube-dilution method of Dornbush and Pelcak.[19] Duplicate tests with each antibiotic were set up in Penassay broth and in yeast extract broth, a 1: 300 broth dilution of the test organism was added, and the tubes were read after seven hours' incubation at 37 C. Aureomycin was found to be the most active, on a weight basis, of five antibiotics tested against our strain of *L. monocytogenes*. The values are given in table 1.

In Vivo Activity of Aureomycin

Rabbit Tests.—In order to study the penetration of aureomycin into the spinal fluid, one dose of 5 mg. of aureomycin per pound of body weight was injected intravenously into each of 3 rabbits. Twenty-four hours after this dose of aureomycin, they were infected intracerebrally with 25 million organisms each. They received the same amount of aureomycin for three more days. All survived, while the control animal died two days after infection.

Another group of 4 rabbits, treated in the same manner at a level of 1 mg. of aureomycin per pound of body weight daily, died within three days.

Mouse Tests.—Twelve 20-Gm. mice were infected intraperitoneally with 0.05 ml. of a 24-hour yeast extract broth culture (360 million organisms). Treatment consisted of two subcutaneous doses of aureomycin given thirty minutes and twenty-four hours after infection. Four mice were treated at a dosage of 10 mg. per kilogram of body weight, 4 at a dosage of 2.5 mg. per kilogram, and 4 were held as con-

trols. The results are reported in table 2. Animals in groups B and C developed a marked conjunctivitis and appeared lethargic within twenty-four hours. Examination of the peripheral blood revealed a striking monocytic reaction which was less severe in the aureomycin-treated animals. Postmortem cultures of the liver and heart blood were all positive for *L. monocytogenes*. Numerous small areas of focal necrosis were observed in the liver, and the spleen presented a mottled appearance. The mice in group A appeared healthy until the fifth day after infection when symptoms developed. The pathologic findings were similar to those in other groups.

TABLE 4

	Days after infection	Neutrophils (%)	Eosinophils (%)	Basophils (%)	Lymphocytes (%)	Monocytes (%)
(A) Aureomycin, 10 mg./kg.	1	33.5	2.0	0.0	57.5	7.0
	2	38.5	1.5	0.0	56.0	4.0
	14	34.0	2.0	0.0	60.5	3.5
(C) Penicillin G, 10 mg./kg.	1	40.0	1.0	0.0	44.5	14.5
	2	21.5	0.0	0.0	39.5	39.0
(E) Untreated controls	1	59.0	0.0	0.0	27.0	14.0
	2	42.0	0.5	0.0	19.5	38.0
(F) Normal mice	..	36.0	1.5	0.5	58.5	3.5

In view of the *in vivo* activity of aureomycin demonstrated in the above test, it appeared desirable to extend the course of aureomycin therapy in a second series and also to compare the activity of penicillin on a weight basis.

Thirty 20-Gm. mice were infected intraperitoneally with 0.05 ml. of an eighteen-hour tryptose-phosphate broth culture (ca. 25 million organisms). Treatment consisted of daily doses of antibiotic administered subcutaneously (beginning 30 minutes after infection) at levels of 10 mg. or 5 mg. per kilogram of body weight for four days. The results are given in table 3. Aureomycin gave complete protection and penicillin showed no activity. All animals in the aureomycin-treated groups appeared normal when the test was terminated on the fifteenth day after infection. Postmortem examination revealed no gross lesions and all liver cultures were bacteriologically sterile.

Animals in the penicillin-treated and control groups developed severe conjunctivitis and appeared lethargic within forty-eight to seventy-two hours. Autopsy revealed multiple minute necroses in the liver, and the spleens were mottled. All liver cultures yielded *L. monocytogenes* in pure culture.

Monocytosis in Experimental Listeria Infections.—Hematologic examinations were made on the tail blood of 2 animals from each group (of the above experiment) at intervals of twenty-four hours, forty-eight hours, and fourteen days after infection. A marked monocytosis was noted in the penicillin-treated and control mice twenty-four hours after infection; in forty-eight hours, the numbers of monocytes were doubled, with a concomitant reduction in the number of granulocytes. This monocytic reaction was not observed in the mice which had received aureomycin therapy. The differential counts are reported in table 4.

SUMMARY

1) A field outbreak of listeriosis in cattle involving 6 animals, two of which died, is described. The diagnosis was established by postmorten isolation of *Listeria monocytogenes* from the brain tissue and by demonstration of specific agglutinins in a recovered animal.

2) Primary isolation cultures yielded no growth of Listeria on blood agar, but thioglycollate broth gave good growth. A suspension of brain tissue held for several weeks under refrigeration yielded increasingly good growth on blood agar.

3) Mice, hamsters, and rabbits were readily infected by giving the culture via the intraperitoneal or intracerebral routes, but chicks were refractory to intraperitoneal injection. Ocular instillation of the culture produced an ophthalmitis in rabbits and guinea pigs.

4) The strain of *L. monocytogenes* showed considerable thermal resistance, withstanding temperatures of 95 C., but not 100 C., for ten minutes.

5) *In vitro* tests showed the strain to be very sensitive to aureomycin (0.04 μg./ml.) and resistant to terramycin, penicillin, chloromycetin, streptomycin.

6) Aureomycin gave 100 per cent protection against experimental infection in mice when the antibiotic was given in daily doses of 5 mg. per kilogram of body weight for four days. Penicillin in 10 mg. per kilo-

gram of body weight doses afforded no protection. A marked monocytosis developed in penicillin-treated and untreated mice but was not observed after aureomycin therapy.

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A Note on Eructation in Sheep as Related to Rumen Fluid Level and Gas Insufflation

R. E. NICHOLS, D.V.M., M.Sc., Ph.D., D.V.Sc.

Madison, Wisconsin

AS IS TRUE with the study of many abnormalities of function, including bloat, an investigation of the cause or causes of a condition is often delayed until some means is found of reproducing the abnormality under circumstances which will allow its further scrutiny. Early in an attempt to study rumen gas absorption under various degrees of pressure, it appeared that there exists a limit to the quantity of gas which an animal will retain in its paunch and that the introduction of further quantities results in increased belching. This interferes with attempts to ascertain the threshold of gas absorption, because one has no control over either the amount or pressure of the gas introduced. It was decided that some means of controlling belching should be attempted, and Dougherty's article[1] suggested the introduction of fluid.

TABLE 1—Belches and Coughs Associated with CO_2 Insufflation (Trial 1)

Time	CO_2 added (cu. ft.)	Belches	Coughs	Visible signs
1:54-1:59	3.16	9		Bloat, rapid respirations.
1:59-2:04	3.64	12	2	Cyanotic. Defacation.
2:04-2:09	5.10	13	7	Dyspneic. Weavy.
2:09-2:14	3.80	21	6	Micturition.
2:14-2:19	2.63	20	5	Very dyspneic.
2:19-2:24	4.53	20	3	Very cyanotic.
6 liters water added to rumen.				
2:25-2:30	4.06	4	6	Animal up and down.
2:30	Relieved pressure and CO_2 applied 5 lb. p.s.i.* without meter.		0	
				Down. Respirations stopped.
2:31	Relieved pressure.			Slowly recovered.

*per square inch.

Twelve sheep were used. A large spirometer, a gas meter, and tanks of compressed gases with control valves were available. Water and gases were introduced either through a 3 in., 16-gauge hypodermic needle in intact animals or through a small pressure plug in those animals which had small rumen fistulas (½ in.). Table 1 depicts the increase in belching as mentioned previously. Of further interest is the associated increase in coughing. Respirations ceased in this animal (trial 1) following the introduction into the rumen of 6 liters of water and CO_2 at 5 lb. per square inch (p.s.i.). Relief of pressure resulted in recovery. A similar increase in belching is recorded in table 2. Seven liters of water added to the rumen failed to stop belching, but 5 lb. p.s.i. of O_2, after the addition of one more liter of water, brought death in six minutes.

TABLE 2—Belches and Coughs Associated with O_2 Insufflation (Trial 2)

Time	Water added	Pressure	Belches	Coughs	Notes
	7 liters				
2:30-2:47		64 lb.*	0	0	
2:47-3:10			18	1	
3:10-3:18		3 lb. p.s.i.**	35	0	
					Micturition.
3:18-3:21			11		
3:25	1 liter				
3:26		5 lb. p.s.i.	1		Very dyspneic.
3:32					Unconscious. Respirations ceased. Death.

*By weight on spirometer. **By compressed O_2. p.s.i. = per square inch.

Table 3 also demonstrates the number of belches following introduction of water and gas into the rumen. In all but two trials, the time of belches in relation to the

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start of insufflation and subsequent death are recorded. In all but 3 of the 9 animals whose belches were timed, eructation began immediately following the beginning of insufflation and continued in 8 of the 9 cases death occurred two minutes after raising to 10 lb. p.s.i. the pressure of the air introduced. Death herein was timed at cessation of respiration and consciousness. Beyond this point none recovered.

TABLE 3—Belches and Death Following Water and Insufflation

Trial (No.)	Weight of animal (lb.)	Water added to rumen (liters)	Gas	Pressure (p.s.i.)*	Time gas started	Belches: Time	Belches: No.	Time of death	Notes
3	55	5	air	2½	.....	.....	1	.....	.....
				5	.....	.....	4	.....	.....
				10	.....	.....	1	2 min.	.....
4	70	7½	H_2	1¼	9:06	9:06-7	8	.....	Needle came out 9:08
					9:10	9:10-12	10	9:12	.....
5	60	7	H_2	1¼	8:44½	8:44½-5	7	8:47	.....
6	70	7	CH_4	1¼	9:52½	9:52½-4	13	9:58	.....
7	...	6	CO_2	5	1:36	.....	10	1:43	Cyanotic mmbs.**
8	...	7	CO_2	3	1:42	1:48-2:09	7	2:19	Long belchings. Insufficient water? Frequent urination.
9	40	5	CO_2	1¼	10:43	10:43-4	15	10:47	Cyanotic mmbs.
10	80	7	CO_2	1¼	10:24½	10:24½-26	13	10:29	Cyanotic mmbs
11	90	6	O_2	2½	9:20	9:20-5	19	9:28	Bright mmbs.
12	120	7	O_2	5	10:21	10:23-4	6	10:27	Bright mmbs. Epistaxis.
13	120	8½	O_2	1¼	9:52	9:53-4	12	9:58	Bright mmbs.

*per square inch; **mmbs. = mucous membranes.

for one-half to five minutes, at which time it ceased rather abruptly. In 2 of the remaining 3 animals, eructation began one minute and two minutes after the beginning of insufflation. The other animal (trial 8) was peculiar in that eructation began six minutes following the beginning of insufflation and continued over several minutes. Instead of a series of short belches, this animal eructed a series of belches of long duration. No attempt was made to measure volumes of belches, flatuses, urine, and feces voided. The volume of water introduced may have been too small. Except for the animal in trial 8, death of those with timed belches occurred in from less than one minute to five minutes following the last belch. Death occurred ten minutes after the last belch in the animal in trial 8. In the animal in trial 3,

Though only from a pilot trial, these data suggest that:

1) Insufflation of the rumen with gas increases belching.

2) Overloading of the rumen (in this case with water), plus insufflation of gases, may not produce an immediate cessation of belching but, with continued insufflation, belching may soon cease and death may follow fairly quickly.

3) Additional investigations are indicated to correlate the continued and suddenly ceased belching (following overloading and insufflation of the rumen) with the motility of the organ under sustained pressures.

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A Study of the Pathology of the Chicken Embryo Infected with a Transmissible Agent Isolated from Sheep Lungs

ALEXANDER M. RAMIREZ, D.V.M., M.S.; M. L. GRAY, A.B., M.S.;
FRANK THORP, Jr., D.V.M., M.S., Ph.D.

East Lansing, Michigan

WITHIN RECENT years, it has been shown by numerous investigators that viruses can be incriminated in the etiology of pneumonia of man and animals. Venkatachalam *et al.*[1] in an analysis of mortality among lambs, showed that pneumonia accounted for 8.7 per cent of the total number of deaths occurring before 56 days of age. This figure was the highest for any of the diseases to which lambs are susceptible. Unpublished data pertinent to this reference indicate that many of these pneumonic lungs failed to yield significant bacterial growth following the usual cultural methods. Thus, a virus etiology was suspected. The purpose of the investigation here reported was twofold: to determine whether a transmissible agent other than bacteria was present in these pneumonic lungs and, if so, to determine if it could be propagated in the embryonating chicken and produce lesions.

Fig. 1—Two embryos, each 19 days old. The control shown at the left is twice the size of the infected embryo.

REVIEW OF LITERATURE

Friedberger and Fröhner[2] were the first to report pneumonia in sheep due to an unknown agent. Mitchell[3] described the clinical and pathologic findings in jagziekte or chronic catarrhal pneumonia of sheep and postulated that this disease was of virus etiology. Cowdry,[4] in a comparison of jagziekte and pneumonia in American sheep, concluded that there was a significant difference between the two diseases and that the former could be transmitted to healthy sheep by exposure to sheep known to be suffering from jagziekte. He too, suggested a virus etiology. Stylianopoulos[5] reported a pleuropneumonia of goats in Greece that seemed to be due to a virus. Experimental infection could be accomplished by pulmonary passage of infected lung tissue or pleural exudate. Blackmore and Bosworth[6] described a disease of sheep in England that was identical in micropathology to the jagziekte described by Cowdry. Bawa[7] reported a contagious pleuropneumonia of goats in India. This was thought to be of virus origin but the agent could not be transmitted to sheep. Dungal[8] concluded

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From the Department of Animal Pathology, Michigan State College, East Lansing.

Dr. Ramirez, at present, is at the Sociedad Ganadera de Junin, Hacienda Pachacayo, Junin, Peru, South America.

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from animal experiments that jaagsiekte was of virus etiology. Morcos *et al.*[9] established that a virus was responsible for a contagious ovine pneumonia existing in Egypt. This virus was found to be pathogenic for rabbits but not for guinea pigs, rats, mice, or pigeons. A formalinized vaccine was prepared from liver and spleen tissue which protected sheep against the infection.

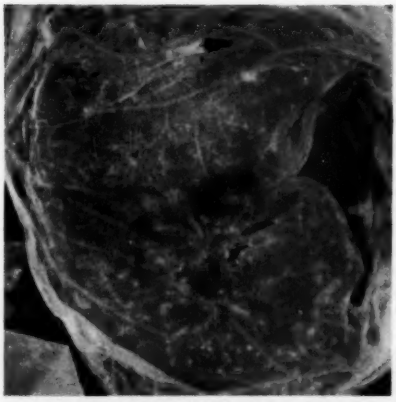

Fig. 2—Chorioallantoic membrane showing edema, hemorrhage, and the presence of "pocks." x 8.

MATERIALS AND METHODS

The agent presented in this report was isolated from a female Suffolk lamb which, at necropsy, showed a bilateral pneumonia with emphysema, congestion, and red hepatization. The lungs appeared dull and displayed marked interstitial edema. The interlobular septums were thickened in the consolidated areas. The trachea and bronchi contained an abundance of dirty grayish exudate and the mucosa of each was congested. Sections of the consolidated and congested areas were removed and fixed in Zenker's fluid.

Microscopically, the lungs showed marked congestion, edema, and perivascular cuffing with lymphocytes and macrophages. The pleura was thickened and adhered to it was a serofibrinous exudate. A few giant cells were seen below the pleura.

Cultures prepared from this lung on sheep blood-agar and tryptose-agar plates failed to show significant bacterial growth.

The method of Burnet[10] was used in an effort to cultivate a transmissible agent from these lungs. Fertile eggs from healthy White Leghorn hens were purchased from the Michigan State College Poultry Department for use in this investigation.

Three grams of tissue from the infected lung were ground in a mortar together with 3 ml. of nutrient broth. The resulting suspension was treated with 1,000 units of penicillin and 0.1 mg. of streptomycin per gram of lung tissue (Rose *et al.*[11]). This use of antibiotics was carried through only the first two egg passages.

Twelve-day-old chicken embryos were inoculated on the chorioallantoic membrane with 0.2 ml. of lung suspension. Some of the embryos were examined each day following inoculation up to and including the eighth day. The membranes showing lesions were triturated in the Waring blendor together with 3 to 4 ml. of broth and employed for subsequent egg passage. Only membranes found to be bacteriologically sterile were used. This procedure was used to carry the transmissible agent through 19 egg passages.

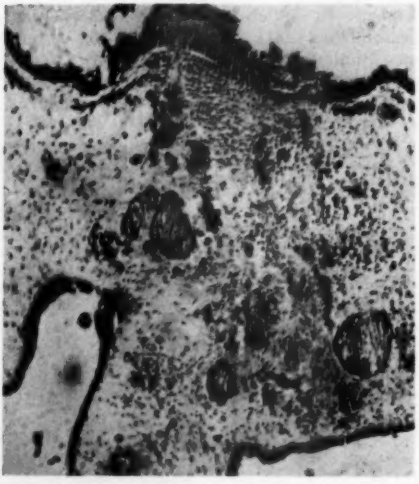

Fig. 3—Thickened chorioallantoic membrane with necrosis of ectodermal layer and epithelial pearls; also extensive leucocytic infiltration. Hematoxylin-eosin stain. x 70.

Helly's solution was poured into the opened eggs and left for one hour, after which the tissues were removed from the embryo and further fixed in Helly's solution for five or six hours. Sections were prepared in the usual manner for histologic study. These were stained with Harris' hematoxylin and eosin or MacCallum's modification of Goodpasture's method for staining bacteria in tissue sections.

RESULTS

The pathologic changes in the embryos

first were noted after the third passage of the agent. Symptoms and lesions in these infected embryos generally developed after three or four days of incubation and can be summarized as follows:

1) Movements were sluggish to the extent that some of the embryos appeared dead.

2) The large blood vessels of the chorioallantoic membranes were less prominent.

3) Death occurred much sooner than in the controls, after exposure to air and lower temperature following opening of the eggs.

4) They were small in size, some being about half that of the controls (fig. 1).

5) The abdominal wall failed to enclose the organs.

6) Lesions were most prominent in the embryos which had succumbed to the infectious agent.

7) The chorioallantoic membrane was congested and showed hemorrhages varying in size from petechiae to ecchymoses. The membrane was thickened, gelatinous, and edematous. Areas of opacity or "pocks" were observed (fig. 2). These areas were either homogenous or granular in appearance. On these patches, particularly on those lying along the large blood vessels, the tissue had proliferated to form yellowish white nodules. These nodules varied in size from 1 to 2 mm. in diameter and showed a dark grayish necrotic spot.

8) The lungs were swollen, more flaccid, and adhered to the thoracic wall by a serofibrinous exudate.

9) Some displayed splenomegaly and, in a few, the medulla oblongata appeared swollen, edematous, and exhibited petechial hemorrhages.

10) Death generally occurred on the seventh day postinoculation.

11) If the embryos survived until hatching age, they appeared too weak to break the shell, and the few which did hatch lived only a short time.

Microscopic Lesions.—The chorioallantoic membrane showed a proliferation of the ectodermal layer and, in some instances, the cells were necrotic (fig. 3). In the mesenchymal layer, formations of epithelial pearls appeared which often resembled the tumor cells of squamous cell carcinoma and, in other instances, Hassall's corpuscles of the thymus. The epithelial pearls appeared as irregular bodies distributed at intervals between engorged blood vessels. These were located in different zones of the mesenchymal layer, but most of them were situated deep in the mesoderm. The larger pearls showed the greatest degree of degeneration and the nuclei of the epithelial cells exhibited pyknosis, karyorrhexis, and karyolysis. The nuclei located in the center of the epithelial nests were round, while those near the surface tended to be oval or elongated and parallel to the periphery (fig. 4). The chromatin of the nuclei, in some in-

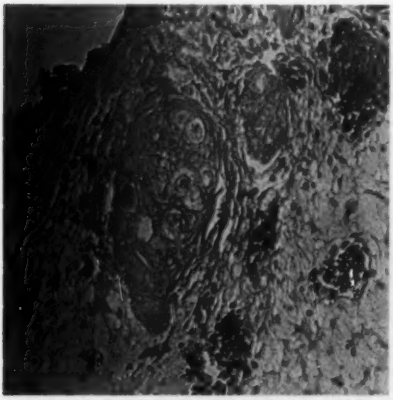

Fig. 4—Chorioallantoic membrane showing epithelial nests with cells in various stages of degeneration, connective tissue around the nests, and congestion of capillaries. Hematoxylin-eosin stain. x 170.

Fig. 5—Chorioallantoic membrane showing a more severe degeneration of the epithelial nests. Balloon degeneration and hyalinization of the epithelial cells. Hematoxylin-eosin stain. x 170.

stances, was located at the periphery. Balloon degeneration of the epithelial cells was noticed (fig. 5). Concentric proliferation of connective tissue was observed around these epithelial pearls. In the older lesions, the mature fibroblasts occupied the periphery of the pearls, while the more immature fibroblasts were located in the zone next to the old, concentrically arranged fibrosed tissue. Other epithelial nests showed complete necrosis and sometimes only a hyalinized zone remained. There was usually a collection of eosinophilic polymorphonuclear leucocytes in the mesoderm near these pearls. Dilatation of the blood vessels, areas of hemorrhage, proliferation of connective tissue and, in some, infiltration of heterophils surrounding the mesenchymal blood vessels were observed. It was significant that all the areas in which the epithelial pearls were found corresponded to the pock lesions located on the surface of the chorioallantoic membranes.

In the early passages of the virus, the embryos showed pneumonia which was characterized by the presence of serous exudate in the bronchi, followed by a thickening of the alveolar walls. The capillaries showed congestion, and perivascular cuffing by leucocytes. Hemorrhages were often seen below the pleura and along its entire length. Edema was present in the interstitial tissue and after each passage of the agent this lesion was more pronounced (fig. 6). Another feature noticed in early stages was the presence of a heavy fibrinous layer of exudate over the thickened pleura. Lymphocytes were present in this fibrinous exudate (fig. 7). Around the alveolar openings, hyalinization of the muscle fibers was noticed. Many of the bronchi showed an increase in thickness of the lamina propria. Epithelial proliferation was probable, since some of the cells seemed to be incarcerated in the form of nests by the connective tissue of the lamina propria (fig. 8). These changes appear to be significant when compared to the lungs of normal chicken embryos.

All of these features were observed in the 19 passages of the agent through the chicken embryo.

DISCUSSION

This report established that there was an agent in pneumonic lungs of sheep which could be propagated on the chorioallantoic membrane of the chicken embryo. The lesions produced in the embryonic lungs further suggested that this agent was pneumotropic.

The characteristic lesions on the chorioallantoic membrane were a proliferative process which first occurred in the form of epithelial pearls and later as degeneration characterized by necrosis and hyalinization. Similar changes were reported by Tang *et al.*[12] and Longley,[13] following exposure

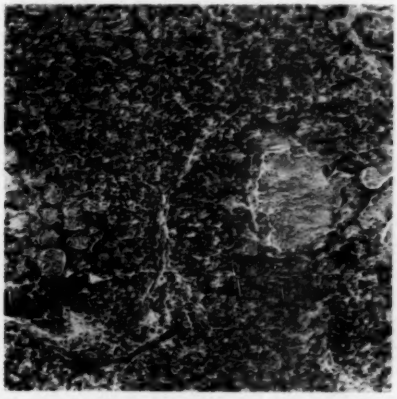

Fig. 6—Infected lung of embryo showing serous exudate involving the entire organ, and thickened alveolar walls.

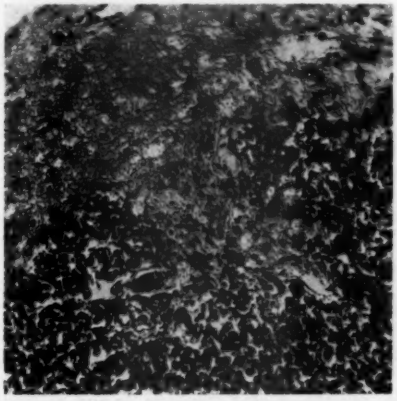

Fig. 7—The pleura is covered with a thick layer of fibrinous exudate. Infiltration of lymphocytes is seen together with the fibrin. Hematoxylin-eosin stain. x 100.

of the chorioallantoic membrane to pleuropneumonia organisms. However, they did not report the presence of lesions in any

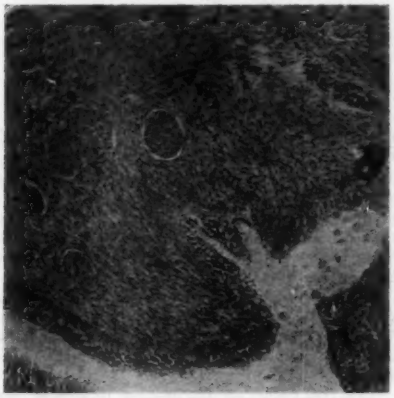

Fig. 8—A bronchus showing increase in thickness of the lamina propria. Epithelial nests in this zone seem to be incarcerated by connective tissue. Hematoxylin-eosin stain. x 170.

of the viscera. Brandly *et al.*,[14] in a study of the response of chicken embryos to tissues of chickens affected with the avian leucosis complex and to tissues of normal birds, reported the presence of epithelial pearls in the mesenchyma. Woodruff and Goodpasture,[15] in a study of fowlpox virus, reported a marked hyperplasia of the ectoderm, thickening of the mesoderm, and hyperplasia of entodermal cells. Inclusion bodies were numerous in the ectoderm, but only a few were found in the endoderm.

In the present investigation, no proliferation of the intradermal layer was seen nor were inclusion bodies observed. Histologically, the lesions appeared to represent primarily the response of the ectodermal epithelium to the agent, with secondary changes in the other layers.

The most characteristic lesion observed in this study was the pneumonia produced in the embryonic lungs. Grossly and microscopically, it resembled the type of pneumonia from which the agent was originally isolated. The inflammatory response in each instance was predominantly characterized by infiltration of lymphocytes and macrophages. These facts suggested that the agent was a pneumotropic type of virus.

SUMMARY

1) A nonbacterial transmissible agent was isolated from pneumonic lungs of sheep. This agent was maintained on the chorioallantoic membrane of developing chicken embryos through 19 serial passages.

2) By passage of this agent in chicken embryos, pneumonia was produced in the embryonic lung.

3) A constant and characteristic macroscopic change was the presence of "pocks" on the chorioallantoic membrane.

4) The most persistent and distinctive microscopic lesions of the chorioallantoic membrane were the epithelial pearls formed by proliferation of the ectodermal cells in the mesenchyma. Necrosis of the ectodermal layer and necrosis and hyalinization of the epithelial pearls were also observed.

5) An apparent interference of growth processes in the chicken embryos was observed.

6) The agent isolated in this study suggested a pneumotropic type of virus.

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Studies on Baby Pig Mortality. VI. Experimental Uremia in Young Pigs

VERA HANAWALT GRACE, Ph.D.; C. C. MORRILL, D.V.M., Ph.D.; ROBERT BUTZOW, M.S.; PAUL HENDREN, B.S.; JESSE SAMPSON, D.V.M., Ph.D.

Urbana, Illinois

IN 1944, MADSEN and co-workers[7] attributed to uremia a mortality of nearly 50 per cent of newly born pigs farrowed on several farms in the vicinity of Beltsville, Md. The diagnosis was based largely on the observations that blood of sick pigs usually showed high uric acid and urea-nitrogen values, and that the kidneys, ureters, and bladder contained variable amounts of an orange-colored precipitate which appeared to consist mainly of uric acid. More recently, Green and associates[4] have reported that uremia developed in young pigs fed a special milk diet.

Since published data concerning biochemical changes in uremia of swine are limited, it was believed worthwhile to obtain more information on this subject. Such information should prove helpful in interpreting the significance of observations made on pigs affected spontaneously with uremia and uremia-like conditions. In this report, data are presented for the nonprotein-nitrogen constituents of blood of young pigs in which anuria and uremia were produced experimentally by obstructing the ureters.

In addition to the biochemical data, a description is given of gross and microscopic changes in the kidneys associated with obstruction of the flow of urine in the ureters.

EXPERIMENTAL

Uremia was produced by ligation of both ureters through flank incisions under general anesthesia with ether or nembutal.® Twenty-four pigs, ranging in age from 3 to 82 days, were used in the experiment. Each baby pig was left with the sow for twenty-four hours following birth. The newly born pigs, as well as the older pigs, were then kept in a metabolism cage and fed *ad lib.* on cow's milk. A small amount of mineral mixture was added to the milk in the hope that it would prevent anemia and possibly diarrhea. Significant anemia did not develop, but diarrhea was not completely prevented. In some of the experiments, ligation of each ureter was done several days apart, while in others both ureters were tied off in the same operation.

Venous blood for analysis was collected over potassium oxalate before surgery and at 24- or 48-hour intervals thereafter. When possible, the last sample was drawn shortly before death.

The technique of Carle and Dewhirst[2] was followed in collecting the blood samples from the anterior vena cava. Rectal temperature was taken at the time of each bleeding. A sharp fall in body temperature was often a helpful sign of approaching death. Frequent observations of clinical manifestations were made.

Nine control pigs were subjected to laparotomy to observe what effect, if any, incision of the peritoneal cavity might have on the blood levels of the nonprotein-nitrogen constituents. In 2 other control pigs, a rubber sleeve was placed around the ureter at the time it was tied off and this was removed twenty-four hours later. In these pigs, normal urination appeared to be re-established within the next twenty-four hours. Nonprotein-nitrogen fractions of the blood were determined before and after removal of the sleeves.

METHODS USED IN CHEMICAL ANALYSIS

The following methods were used in the determinations on the blood: total nonprotein nitrogen, Koch and McMeekin;[6] urea nitrogen, Van Slyke and Cullen;[5] uric acid, Folin;[6] creatinine, Folin-Wu;[6] and allantoin, Christman, Foster, and Esterer.[3] Determinations for allantoin were not made until approximately one half of the trials had been completed, because of difficulty encountered with the method. Correction was made in the method for the error due to uric acid but not for that due to ergothioneine, because of failure to obtain a sample of this compound to be used as a standard. The error due to ergothioneine is about one twentieth of the total ergothioneine content in the blood of mature swine. In 7 mature animals, the average content was found to be 7.6

From the Department of Veterinary Physiology and Pharmacology, College of Veterinary Medicine, University of Illinois, Urbana.

Contribution from the Illinois Agricultural Experiment Station as a collaborator under North Central Regional Agricultural Experiment Station coöperative research project entitled "Death Losses in Young Pigs."

TABLE I—Experimental Uremia in Young Pigs—Nonprotein-Nitrogen Fractions in the Blood

Experimental pigs												
		Blood nonprotein-nitrogen fractions at time of bilateral ligation					Blood nonprotein-nitrogen fractions prior to death					
Pig (No.)	Age at time of ligation of 2nd ureter (days)	NPN (mg. %)	Urea N (mg. %)	Uric acid (mg. %)	Creatinine (mg. %)	Allantoin (mg. %)	NPN (mg. %)	Urea N (mg. %)	Uric acid (mg. %)	Creatinine (mg. %)	Allantoin (mg. %)	Survival (days)
534	3	76.92	35.75	0.54	1.38	...	201.34	145.20	5.30	2.53	....	1
650*	6	53.33	12.10	0.53	1.05	...	111.86	64.90	6.17	4.05	....	1
652*	6	60.59	24.20	0.67	0.90	...	160.00	114.40	5.78	3.71	....	1
1382*	6	45.91	14.30	1.50	1.32	2.63	106.19	73.70	2.28	5.02	13.49	2
1383*	6	35.01	8.80	0.38	1.28	3.07	140.35	88.68	2.35	7.45	15.17	3
1384*	6	31.14	6.60	0.22	1.67	2.56	115.94	67.10	1.54	5.38	14.24	2
103	8	43.32	7.48	0.27	1.60	1.91	148.14	70.40	2.11	5.77	6.50	3
105	8	37.15	6.38	0.47	1.66	1.44	147.23	107.80	7.01	6.00	12.46	4
801	10	59.11	22.57	0.76	1.26	...	260.11	235.84	6.53	5.33	....	2
807	10	41.66	4.97	0.64	0.93	...	398.01	270.60	3.02	9.88	....	5
950*	11	45.25	12.10	0.39	1.03	1.52	255.31	211.20	5.21	8.29	15.26	4
424*	12	45.11	13.20	0.69	1.80	2.05	78.81	38.50	0.80	4.51	9.34	2
428*	12	40.00	13.20	0.56	1.59	1.55	165.51	134.20	1.36	8.76	15.20	4
425*	13	39.34	3.30	0.50	2.19	2.02	75.23	41.27	0.20	7.07	14.63	4
1274*	25	42.40	10.47	0.55	1.12	1.32	169.49	135.87	2.19	6.57	24.67	3
1277*	25	48.98	8.27	0.59	1.04	1.18	178.59	135.87	1.73	5.92	16.67	2
808	32	28.57	4.40	0.40	1.69	...	130.43	99.00	0.58	6.87	....	4
804	38	33.24	6.60	0.33	1.02	...	105.10	80.74	0.71	10.62	....	4
761	52	31.24	19.80	0.23	2.41	...	201.34	145.20	1.29	10.80	....	7
645	72	40.68	12.74	0.56	1.44	...	183.90	120.00	2.67	8.65	....	5
651	72	43.48	9.24	0.62	1.88	...	194.64	143.00	1.60	10.74	....	6
760	80	54.05	9.90	0.55	1.59	...	366.97	289.30	0.73	9.97	....	12
100	81	40.72	11.00	0.42	1.97	...	368.09	310.20	1.31	15.67	....	11
647	82	54.52	4.40	0.61	1.59	...	218.18	181.50	0.06	4.84	....	12
Average		44.65	11.74	0.54	1.47	1.93	186.70	137.69	2.61	7.27	13.14	4.33

*Both ureters ligated simultaneously.

mg. per 100 ml. of blood.[5] No data were found for the constituent in young pigs.

RESULTS

Chemical Data.—Data in table 1 show the age of each pig when the second ureter was ligated, the number of days each pig survived following bilateral ligation, and values for blood nonprotein nitrogen, urea nitrogen, uric acid, creatinine, and allantoin before and after anuria and uremia were produced. Three pigs that were 80 or more days old when the second ureter was tied off lived eleven to twelve days. In the younger pigs, the time of survival after double ligation was much shorter than this. The effects of shock may have been greater in the younger than in the older pigs, but both age groups withstood equally well the effects of ligation of only one ureter.

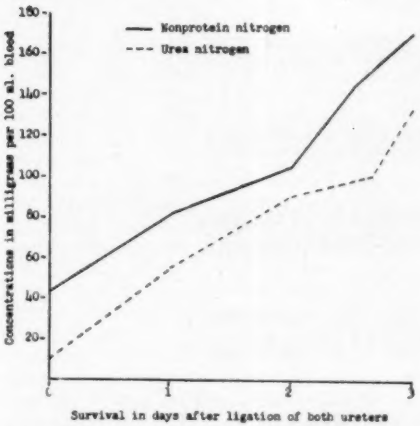

Fig. 1—Concentrations of nonprotein nitrogen and urea nitrogen in the blood of a 25-day-old pig from time of ligation of both ureters until death.

Elevation of the concentrations of the various nitrogenous fractions began immediately after ligation of the second ureter, *whereas ligation of only one ureter did not cause an abnormal increase in any blood constituent* (table 1). Very young pigs developed uremia rapidly following bilateral ligation and, even though they died within one or two days, the nitrogenous concentrations of most components were very high when death occurred. However, the most pronounced increase of nitrogen occurred in pigs that lived five or more days. As uremia developed, the nonprotein-nitrogen concentration rose from an average of 44.65 to 186.40 mg. per 100 ml. of blood, urea nitrogen increased from a normal average of 11.74 to 137.69 mg. per 100 ml. of blood, and creatinine rose from 1.47 to an average of 7.27 mg. per 100 ml. of blood.

The uric acid values were of special interest. The average prior to bilateral ligation was 0.54 mg. per 100 ml. of blood for the 24 pigs, and just before death this figure rose to an average of 2.61 mg. per 100 ml. of blood. However, in pigs surviving the shorter period, as represented by the younger group, there was, on the whole, a greater increase in uric acid concentration than in older animals. Pig 105, which was 8 days old when both ureters were tied off, survived four days and had the highest concentration of uric acid, *i.e.*, 7.01 mg. per 100 ml. of blood, whereas pig 647, which was 82 days old, showed an actual decrease in uric acid concentration just before death, when the initial value of 0.61 mg. per 100 ml. dropped to 0.06 mg. per 100 ml. of blood. Since so few determinations were made of

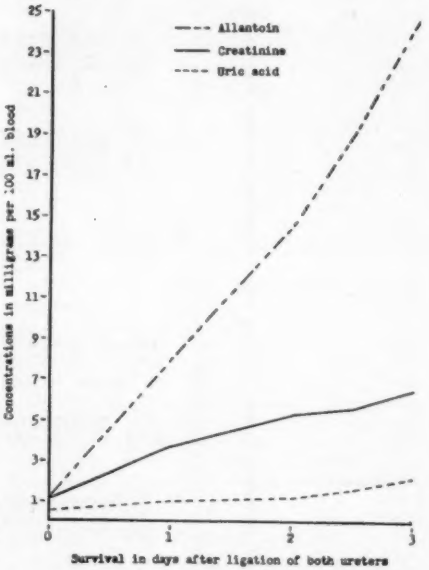

Fig. 2—Concentrations of allantoin, creatinine, and uric acid in the blood of a 25-day-old pig from time of ligation of both ureters until death.

TABLE 2—Experimental Uremia in Young Pigs—Nonprotein-Nitrogen Fractions in the Blood

Pig (No.)	Age at time of ligation (days)	Concentrations before ligation					Concentrations 24 hours after ligation				Concentrations 4 days after release of ureters			
		NPN (mg. %)	Urea N (mg. %)	Uric acid (mg. %)	Creatinine (mg. %)	Allantoin (mg. %)	NPN (mg. %)	Urea N (mg. %)	Uric acid (mg. %)	Creatinine (mg. %)	Allantoin (mg. %)	NPN (mg. %)	Urea N (mg. %)	Uric acid (mg. %)	Creatinine (mg. %)	Allantoin (mg. %)
Experimental pigs—ureters released 24 hours after ligation																
212*	26	40.95	2.75	1.28	1.27	0.50	71.00	30.00	0.92	4.71	5.46	58.28	15.40	0.50	1.71	0.99
216*	33	32.43	8.80	0.00	1.50	1.25	83.76	50.88	1.53	4.96	8.49	46.15	14.30	0.68	1.86	0.87
Control pigs		**Concentrations before laparotomy**					**Concentrations 1 day after laparotomy**					**Concentrations 5 days after laparotomy**				
3231	3	46.54	9.90	0.44	1.76	1.63	60.28	9.33	0.40	1.43	1.36	56.91	14.30	0.32	1.48	0.76
3232	3	54.93	3.30	0.28	1.65	1.83	59.95	6.38	0.14	1.32	...	69.95	...	0.23	1.32	...
3233	3	54.93	4.40	0.20	1.65	1.38	59.59	9.33	0.12	1.59	...	57.56	14.30	0.28	1.32	0.33
805**	10	42.70	33.57	0.83	1.36	...	38.09	7.23	0.48	1.32	...	43.79	17.60	0.89	1.46	...
3001	12	48.43	5.50	0.62	1.91	1.72	43.41	7.08	0.20	1.81	0.97	49.72	11.00	0.39	1.59	0.55
3002	12	46.54	2.64	0.28	1.32	1.55	56.26	6.38	0.34	1.54	...	54.93	7.70	0.44	1.48	0.55
1501	38	41.67	8.80	0.49	1.51	1.29	54.79	13.20	0.52	1.33	...	45.72	4.40	0.44	1.46	0.38
1502	38	46.34	6.60	0.61	1.53	1.25	45.98	9.90	0.48	1.57	1.03	46.14	4.30	0.53	1.35	0.71
1503	40	44.44	11.00	0.69	1.64	0.76	40.96	15.40	0.73	1.51	0.66	42.55	12.63	0.71	1.47	0.54
Average		47.39	9.52	0.49	1.65	1.43	50.26	9.36	0.38	1.49	0.97	52.61	10.65	0.48	1.42	0.59

*Both ureters ligated simultaneously. **Determinations on pig 808 made seven days after laparotomy.

the allantoin content of the blood of these pigs, the average normal concentration of this constituent was not established in the various age groups, and thus evaluation of these data is difficult. There is an implication, however, that the older pig may be better able to convert uric acid to allantoin than the younger pig.

Christman, Foster, and Esterer,[3] in 1944, reported a corrected allantoin level of 0.33 mg. per 100 ml. of blood in 7 hogs. The average allantoin concentration in 11 determinations made in the course of the experiments described in this report was 1.93 mg. per 100 ml. before ligation of both ureters and 13.14 mg. per 100 ml. of blood just before the pigs succumbed, showing that this constituent increased markedly in uremia.

Figures 1 and 2 show graphically the pronounced rise in the five nitrogenous constituents of the blood of pig 1274 as experimental uremia developed. This pig, which was 25 days old when both ureters were ligated, lived three days.

In table 2, data are shown for pigs 212 and 216 in which a rubber sleeve was placed under the ligatures at the time of ligation so that the ureters could be released twenty-four hours later. Nonprotein-nitrogen fractions in the blood of these pigs are shown prior to ligation, twenty-four hours after ligation, and four days following removal of the rubber sleeves. Both pigs began to urinate within twenty-four hours after the sleeves were removed and, at the end of forty-eight hours, there was no evidence of albumin in the urine. The data show that there was a marked increase in all five nitrogenous constituents determined within twenty-four hours after both ureters were tied off. However, four days after release of the ureters, all five nitrogenous fractions had returned to normal levels. Thus, the injury resulting from obstruction of urine flow for twenty-four hours apparently was not severe enough to cause permanent functional impairment of the kidneys in these pigs.

Data for 9 control pigs, varying in age from 3 to 40 days, showing the effects of laparotomy on the nonprotein-nitrogen constituents are also presented in table 2. There was no pronounced increase in the concentration of any of the nitrogenous fractions one day after laparotomy; five days later all values were still normal. Thus,

incision of the peritoneal cavity of pigs of these ages did not produce a significant change in the nitrogen values.

Symptoms associated with uremia caused by obstructing the flow of urine in the ureters were apathy, occasional diarrhea, drowsiness, weakness, and coma. Pigs dying within twenty-four to forty-eight hours after bilateral ligation manifested fewer symptoms than those that survived longer intervals. In the former, there was a rapid decrease in body temperature followed by coma and death. In both older and younger pigs, a progressive failure of appetite accompanied the fall in body temperature. Vomiting and convulsions were not observed in any of the pigs affected with uremia.

Pathologic Observations.—The inevitable result of ligation, of course, was accumulation of urine in the kidney and ureter above the ligature. This resulted, ultimately, in a cystlike enlargement of the kidney. In those cases in which unilateral ligation was performed first and the function shifted to one kidney for a considerable time, the kidney proximal to the first ligation became little more than a thin-walled multilocular cyst (fig. 3). The functional kidney concurrently underwent some compensatory hypertrophy and functioned satisfactorily until its ureter also was ligated. When both ureters were ligated simultaneously, the degree of distention in each was about equal (fig. 4) and was intermediate between that of the two kidneys in cases in which a considerable interval elapsed before the second ureter was ligated. The relative thicknesses of the renal parenchyma are illustrated in fig. 5 and 6.

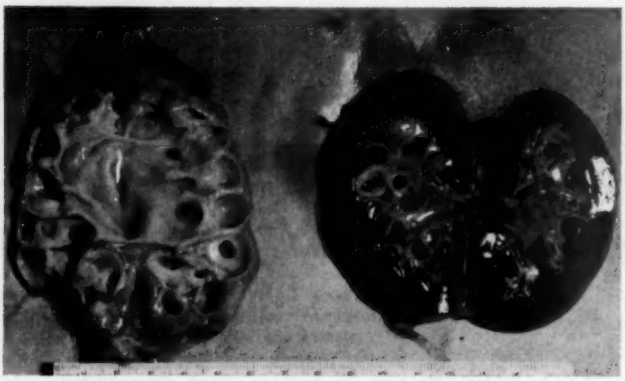

Fig. 3—Kidneys from pig 645. The ureter of the left kidney was ligated at 26 days of age and the right one at 72 days. The left kidney is little more than a multilocular cyst; the right one has undergone some hyperplasia. Death occurred five days after ligation of the right ureter.

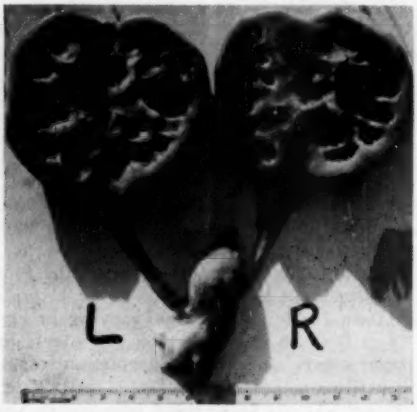

Fig. 4—Kidneys from pig 1383. Both ureters were ligated at 6 days of age and death occurred three days later. Note the approximately even distention of the two kidneys.

The histopathologic picture likewise varied with the time elapsing between ligation and death. When only one ureter was ligated and the opposite kidney remained functional, the kidney proximal to the ligation ultimately became enlarged to a variable degree and the parenchyma atrophied almost to the point of disappearance, leaving chiefly the interstitial or connective tissue framework (fig. 7). When the second ureter was ligated, uremia followed and there was acute damage to the paren-

chyma. This consisted of rather diffuse necrosis which was manifested particularly by pyknosis and by some loss of outline or structure of the tubule epithelial cells (fig. 8). This probably resulted partly from pressure and partly from exposure to accumulated toxic substances in the urine. The glomerular space usually was enlarged by virtue of the fact that the capillary tuft appeared shrunken (fig. 8).

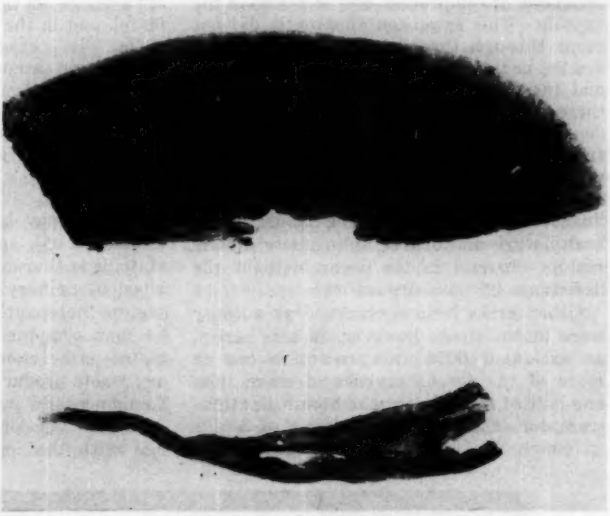

Fig. 5—This shows the comparative thickness of the renal parenchyma in a pig in which one ureter was ligated approximately six weeks earlier than the second. The one whose ureter was ligated first (above) became markedly atrophic while the other became hypertrophic. Compare with fig. 3.

Hemorrhage was commonly seen in the kidneys. On the surface, it was sub- and intracapsular and tended to occur more often near the poles than elsewhere, although it was quite diffuse at times. Ecchymoses and sometimes more voluminous hemorrhages were present in the pelvis. In one kidney there was massive hemorrhage, in a second there were several small blood clots, and in 6 pigs (10 kidneys) the fluid was diffusely blood tinged to some degree. In general (18 pigs, 31 kidneys), the fluid in the pelvis was amber in color and varied from almost clear to distinctly turbid. In three kidneys from 3 separate pigs, the kidney contained pus. Two of these were ligated on one side at a time (at 28/52 days and 26/80 days), and the third was subjected to bilateral ligation at eighty-two days. Bacteriologic examination of the pus revealed the following: in pig 761—*Salmonella choleraesuis, Escherichia coli,* and Proteus sp.; in pig 760—Pseudomonas sp., a hemolytic Micrococcus, and Proteus sp.; and in pig 647—an *alpha*-hemolytic Streptococcus and Proteus sp. Histopathologic examination revealed purulent inflammatory reactions in these three kidneys. In addition, 24 kidneys from 16 pigs showed some degree of definite inflammatory

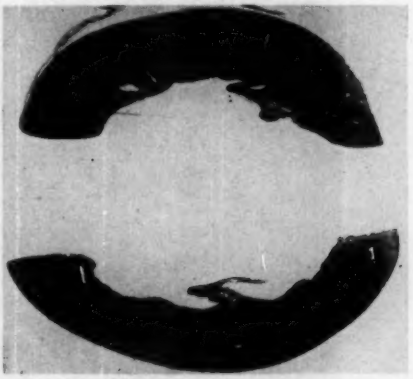

Fig. 6—This shows the relative thickness of the renal parenchyma in a pig whose ureters were ligated simultaneously. There is uniform distention comparable to that shown in fig. 4.

change, characterized usually by necrosis, edema, some hemorrhage, and leucocytic infiltration, particularly in the pelvis and medulla. The leucocytes were predominantly neutrophils. In about one third of these cases, inflammation resulting from direct extension of the process was seen also in the capsule. This extension apparently did not occur through the medulla and cortex but via the connective tissue of the renal sinus and thence to the capsule. Since most of these kidneys showed no microscopic evidence of inflammation as distinguished from edema, bacteriologic examinations were not made. It would seem likely, however, that the physical and chemical influences induced by ligation would be of such nature and degree as to incite inflammatory changes in the organ without the assistance of microörganisms.

Other gross lesions observed at autopsy were inconsistent; however, in this series, an excess of fluid was present in one or more of the serous cavities of more than one half of the pigs in which both ligations were done the same day and in 2 of 3 pigs in which the second ligation followed the first by only one day. When one ureter was ligated a week or more before the second, no significant excess was found. The fluid was amber in color and often contained a small amount of fibrin. It varied in amount from about 10 to 120 ml., with an average in the pleural cavity of about 60 ml. and in the peritoneal cavity of about 45 ml. In 5 cases, there were both hydrothorax and ascites; in 4, there was hydrothorax without ascites; in 1, there was ascites without hydrothorax. All those showing the effusions were relatively small pigs, not more than 4 weeks old.

DISCUSSION

Uremia may be caused by a number of influences but, as Cantarow and Trumper[1] state, it is characterized by retention in the blood of urinary waste products. According to these authors, "uremia . . . refers to that symptom complex which is associated with retention in the blood of urinary waste products and which is dependent fundamentally upon marked interference with the functional activity of the kidneys and with the consequences of such inter-

Fig. 7—Atrophy to the extent of almost complete disappearance of the parenchyma of a kidney from pig 651 whose ureter was ligated at 28 days of age and the second one allowed to carry on the function until 72 days of age. Compare with fig. 3 and 4. Hematoxylin and eosin stain. x 140.

ference. It does not include so-called prerenal azotemia (nitrogen retention), which may resemble true uremia in several respects but which depends upon factors which may operate in the absence of renal disease or serious renal functional impairment."

Kolmer[6] also lays stress upon the retention in the blood of urinary waste products in uremia. He says, "True uremia is the terminal manifestation of renal failure associated with retention in the blood of urinary waste products. Pseudo-uremia or false uremia is due to intracranial circulatory disturbances and has been so designated because some of the signs and symptoms resemble those of true uremia."

On the basis of the views expressed by Kolmer and by Cantarow and Trumper, a diagnosis of uremia may not be justified if nitrogen retention in the blood is the only criterion. Furthermore, as these authors point out, elevation of the blood nonprotein-nitrogen compounds is not believed to be responsible in itself for the symptoms of uremia because these substances are thought to be relatively nontoxic. A marked increase in the blood-creatinine fraction, however, is usually looked upon as a grave sign of renal failure. Thus, a diagnosis of uremia should be carefully considered, particularly with reference to its differentiation from prerenal azotemia.

The data shown in table 1 seem to indicate that the uric acid fraction increases in uremia in pigs up to 10 or 12 days old, while in older pigs the increase is in the allantoin fraction.

In this connection, attention should be called to the statement by Nelson with respect to the significance of uric acid infarcts in the kidneys of the human infant. Nelson[9] states that "In the newborn infant there may be uric acid infarctions of the kidney, producing attacks of abdominal pain, which otherwise are without importance. The mere discovery of urates or of an excess of uric acid in the urine has no particular significance."

On the other hand, the rapid increase in the urea nitrogen and creatinine fractions in the blood of young pigs with complete

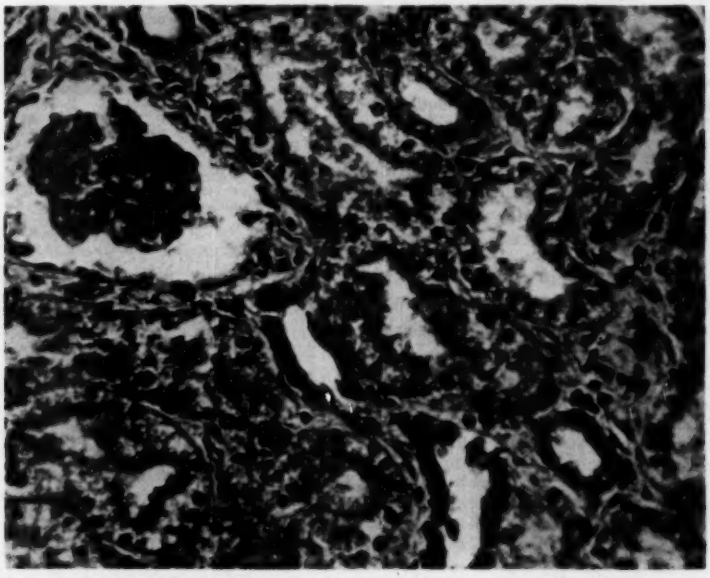

Fig. 8—Section of kidney from pig 651 following ligation of the second ureter at 72 days. Note the evidence of acute or recent damage to the renal tubule cells. Necrosis is denoted by pyknosis, particularly, and by some loss of cytoplasmic boundaries. Note also the shrunken glomerulus and increased glomerular space. Hematoxylin and eosin stain. x 300.

obstruction of the ureters illustrates the extreme importance of the kidneys in maintaining homeostasis. In 1948, McCance[8] pointed out that the function of an organ may change as development occurs. According to McCance, homeostasis is maintained *in utero* in part by the placenta, but a few minutes after birth the kidney must take over and from then on it must excrete nearly all of the nitrogenous end products of the body.

It is significant that in the experiments with pigs described in this report no marked changes occurred in the nonprotein-nitrogen fractions of the blood when only one ureter was obstructed. Furthermore, obstruction of one ureter did not jeopardize the life of the pig and apparently caused no serious functional disturbance.

Temporary obstruction (24 hours) of both ureters caused a rapid increase in the nonprotein-nitrogen constituents of the blood but evidently caused no permanent functional impairment. Since these observations were made on only 2 pigs, it would not be safe to conclude that even temporary obstruction of urine flow would not have some harmful effect.

As would be expected, laparotomy did not appear to affect the uriniferous organs.

The cellular changes in the kidneys associated with obstruction of the ureters were severe and emphasize the dire consequences that follow when the flow of urine is completely checked in young pigs.

It is interesting to note that vomiting and convulsions were not observed in any of the pigs in which uremia was produced by obstructing both ureters.

SUMMARY

In experimental anuria and uremia produced in 24 pigs by ligating both ureters, the blood concentrations of nonprotein nitrogen, urea nitrogen, uric acid, creatinine, and allantoin were greatly increased. The increase in uric acid was more pronounced in the younger pigs than in the older pigs, indicating that the former may not as yet have been physiologically adapted to convert uric acid to allantoin.

Obstruction of one ureter caused no marked changes in the nonprotein-nitrogen fractions of the blood and did not jeopardize the lives of the pigs even though the affected kidney was destroyed.

Complete obstruction of the flow of urine following bilateral ligation of the ureters was associated with severe anatomic and functional changes in both kidneys.

The chief symptoms associated with anuria and uremia in these pigs were apathy, drowsiness, weakness, and coma. Vomiting and convulsions were not observed.

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The Pathology and Symptomatology of Transmissible Gastroenteritis

W. W. BAY, D.V.M., M.S.; L. P. DOYLE, D.V.M., M.S., Ph.D.;
L. M. HUTCHINGS, D.V.M., M.S., Ph.D.

Lafayette, Indiana

Review of Literature

Transmissible gastroenteritis in pigs was first described by Doyle and Hutchings.[3] They observed sporadic outbreaks of a disease in Indiana characterized by diarrhea, vomiting in some cases, rapid loss of weight, and a high death rate in baby pigs. Death losses were limited largely to baby pigs; older swine became infected, but rarely died. The older animals usually developed a diarrhea and a loss of appetite, and in some cases vomited. They also reported cases where the disease apparently spread from baby pigs and brood sows to shoats on the same farm. Doyle and Hutchings also reported that in some cases the disease failed to recur on affected farms where sows, whose litters had been affected, produced additional litters within the same year. The pathology described by them included gastritis, enteritis, congestion of the mesenteric blood vessels, and degenerative changes of the kidney. All of the above features were not necessarily seen in each pig. The most constant finding was a highly fluid intestinal content having a whitish, yellowish, or greenish color. They differentiated this disease from hog cholera by exposing hog cholera-susceptible pigs to the gastrointestinal contents of gastroenteritis-infected pigs without producing hog cholera.

Feenstra *et al.*[4] reported findings similar to those of Doyle and Hutchings. They also reported a slight rise in temperature the first twenty-four hours with a gradual decline until death.

Whitehair *et al.* [11] described a gastroenteritis in pigs resulting in severe death losses. Mortality was highest in pigs less than two weeks old. Older shoats and brood sows were affected. The older animals showed symptoms of severe diarrhea, vomiting, and rapid weight loss, with recovery in two or three days. Pathologic changes noted in pigs 1 to 4 weeks old included inflammatory changes of the mucosa and submucosa of the terminal part of the small intestine and to a lesser degree in the stomach and large intestine. Degenerative changes were also noted in the liver and kidneys.

Doyle and Hutchings,[3] as well as Feenstra *et al.*,[4] were able to transmit the disease by feeding small amounts of ground gastrointestinal tract to young pigs, or by putting diseased pigs in contact with healthy ones. They were also able to produce the disease with bacteria-free filtrates of the gastrointestinal tract.

Bay *et al.*[1] described a change in the blood cells of pigs affected with this disease. They reported a 49 per cent increase in the leucocytes on the fourth day after exposure. The neutrophils were increased and the lymphocytes decreased. There was a preponderance of juvenile type neutrophils present on the fourth day after exposure.

Heidebrecht *et al.*[7] were unable to demonstrate a toxic substance or nutritional deficiency in the milk of sows nursing sick pigs when the milk alone was fed to weanling rats up to four weeks.

Cheever and Mueller[2] reported an epizoötic disease of sucking laboratory mice. The typical disease usually appeared when the mice were between 10 to 15 days old. It was manifest by a diarrhea which varied from passage of a yellowish watery stool to a condition recognized only by the presence of fecal material adhering to the under surface of the tail. This nearly always proved fatal in mice 10 to 15 days old. The mother mice remained healthy in appearance. The disease was transmitted by contact and by exposure to filtrates and crude suspensions.

Pappenheimer and Enders[10] reported intranuclear inclusions in 75 per cent of the mice suffering from a spontaneous diarrheal disease. Such inclusions were confined to the epithelial cells of the small intestine and to the intestinal and mesenteric lymphadenoid tissue.

Gordon *et al.*[5] observed numerous outbreaks of gastroenteritis in adult human beings in New York State institutions. The disease was characterized by sudden onset, profuse diarrhea, vomiting on occasion, and lack of fever. The symptoms ordinarily persisted for about three days. Bacterial agents that are known to cause enteric diseases were not found. The illness was reproduced and transmitted in a series of human volunteers by filtrates.

From the Department of Veterinary Science, Purdue University, Lafayette, Ind.

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Light and Hodes[5] isolated, from cases of infantile diarrhea, a filterable agent which regularly caused diarrhea in young calves. The calves showed extensive hyperemia of the large and small intestines, accompanied by edema of the intestinal walls. Microscopic observations of the large and small intestine showed polymorphonuclear cells at the base of the crypts and elsewhere in the mucosa. In other cases, these cells were found infiltrating the submucosal lymphoid tissue. No inclusion bodies were seen.

Experimental Procedure

The clinical and gross pathologic findings are based on observation of 552 baby pigs which were experimentally infected with transmissible gastroenteritis and of 15 naturally infected herds. The naturally infected herds ranged in size from 8 to 40 brood sows. Microscopic studies were made on the tissues from 20 artificially induced cases in pigs 3 to 5 days of age. These pigs were each fed 2 cc. of ground gastrointestinal tract and its contents from natural transmissible gastroenteritis-infected pigs. At the time of necropsy, specimens of several tissues and organs were taken from each pig. Five healthy pigs, 3 to 5 days old, were killed and tissues taken in order to make comparisons between the tissues from diseased and healthy animals. The tissues from each pig were identified and fixed in 10 per cent formalin. The sections were processed and embedded in paraffin.[8] The sections were stained by hematoxylin and eosin.[9] Scrapings from the tongue were prepared and stained for inclusion bodies according to the method described by Goss *et al.*[6]

Results

Clinical Observations.—Where young pigs were farrowed and housed together, transmissible gastroenteritis was usually a very acute and destructive disease involving all or nearly all of the baby pigs in the herd. The course of the disease was usually short. Young pigs died within two to seven days after exposure, and older swine usually recovered in seven to ten days. The incubation period ordinarily varied from one day to a week, although longer periods have been observed. The disease was observed to begin either in the sow or in the pigs in different herds. Outbreaks have been observed following the purchase of exposed or infected pregnant sows. Other cases have occurred on farms where no new swine had been purchased recently. In most cases, the disease did not spread to neighboring herds.

When the disease was first noticed in a sow, she usually went off feed, vomited, and began to scour. The feces was of normal color in the beginning but, as the disease progressed, it usually changed to a lighter color. There was anorexia, considerable loss of weight, dehydration, and weakness in sows that continued to scour. The sows ceased to lactate, and some pigs appeared to have starved to death. Ordinarily, when older animals in the herd became infected, a large per cent of the adults showed some evidence of the disease.

The pigs, especially those under 1 week of age, died rather rapidly. They usually developed a diarrhea, vomited, became dehydrated, emaciated, comatose, and died. The feces of infected pigs had a marked odor and were very fluid, varying in color from a whitish to yellowish green. About 90 to 100 per cent of the pigs less than 1 week old that showed symptoms died. The death rate gradually decreased until, at 3 weeks of age, approximately 40 to 60 per cent of the pigs recovered. Occasionally, the disease has been observed to cause a few deaths in shoats and breeding stock.

Gross Pathology.—The macroscopic changes varied a great deal depending largely upon the age of the pigs. In pigs that died at 3 to 4 days of age, an atonic intestine containing very fluid contents varying in color from a whitish to yellowish green was the most constant finding; however, hyperemia of the stomach and intestine was commonly found. In most pigs, the stomach was filled with curdled milk. The kidneys frequently contained urates and showed some evidence of degeneration.

As the pigs became older, the disease ran a longer course. The mucosa of the stomach and intestine was often found engorged with blood and frequently areas of necrosis developed. These changes in the stomach and intestine were easily seen from the serous sides of these organs. There was seldom any evidence of free hemorrhage from the mucosa of the stomach or intestine, as judged by the appearance of the intestinal or gastric contents. In most cases, the mesenteric blood vessels were engorged with blood. The cortex of the kidney was rather light in color, and it was difficult to differentiate the various portions. The medullary rays were often markedly congested and easily seen by the unaided eye.

Microscopic Pathology.—The microscopic changes in the gastrointestinal tract of a diseased pig varied with the severity of

the infection and the length of life after onset of the disease.

Stomach.—The microscopic changes in the mucosa of the stomach varied from a congestion of the terminal blood vessels to necrosis of the epithelium deep in the gastric crypts. The mucosa of the stomach of pigs which became infected and died when 2 to 4 days old did not show the pronounced necrosis and extreme engorgement of blood vessels seen in pigs which lived longer.

The surface epithelium was frequently lost and the terminal blood vessels were extremely congested. The nuclei of the epithelial cells showed karyorrhexis and pyknotic changes in some cases. Patchy necrosis of the epithelium lining the gastric crypts began at the surface and, in some cases, extended to the bottom of the crypts. Some of the adjacent crypts did not show much change.

In some of the areas with extensive necrosis and necrobiotic changes, polymorphonuclear leucocytes were prevalent. Round cell infiltration (leucocytes and monocytes) was a common observation in those areas where damage to the mucosa was pronounced. The blood vessels of the submucosa and muscular layers were engorged with blood resulting in some thickening in these areas.

Small Intestine.—The changes in the small intestine were similar to those in the stomach. The mucosal changes varied from congestion of the terminal blood vessels to denudation and necrosis of one-third to one-half the depth of the villi. As a general rule, there was congestion of the blood vessels of the mucosa and some damage to the surface epithelium. The villi in many cases were swollen and had a very pronounced club-shaped appearance as a result of hyperemia and edema. The nuclei of some epithelial cells showed pyknotic and karyorrhectic changes. In areas where the epithelium was undergoing necrosis and necrobiotic changes, polymorphonuclear leucocytes were found. Round cell infiltration of the mucosa, especially near the muscularis mucosae, was a frequent finding. This round cell infiltration seemed to be rather patchy and occurred in those areas showing more pronounced changes in the mucosa. There was some hemorrhage in the mucosa and well-marked congestion of the blood vessels of the submucosa and muscular layers.

Colon.—The microscopic changes in the colon were similar to those in the other portions of the gastrointestinal tract. However, there was a more pronounced round cell infiltration of the mucosa and submucosa. In some parts of the colon, one third to one half of the surface epithelium had disappeared and had been replaced by a diphtheritic exudate. The remaining portion of the mucosa was rather diffusely infiltrated with lymphoid cells. Some of the epithelial cells lining the crypts were normal in appearance, whereas others were undergoing necrobiotic changes. A few polymorphonuclear leucocytes were seen scattered throughout the mucosa. This extensive change in the mucosa was not as marked in some areas of the colon as in others, but some destruction and desquamation of the epithelium was commonly seen.

The blood vessels of the mucosa, submucosa, and muscular layers were congested and, in some cases, free hemorrhage was observed.

Kidney.—In a majority of the kidneys examined, nephrosis was observed. Cloudy swelling of the tubular epithelium, especially in the convoluted tubules, was a nearly constant finding. In some cases, the cellular swelling was so marked that the lumens of the tubules were completely closed. The convoluted and collecting tubules in some kidneys were greatly dilated. This would suggest an occlusion of the tubules near the pelvis, with retention of the excreted fluids. Small areas of focal necrosis of the epithelium of the convoluted tubules were observed occasionally. The capillaries of the tufts of some glomeruli were greatly congested. A few hyaline casts were found in the tubules of some kidneys. The blood vessels of the medullary rays were markedly congested and, in a few instances, some evidence of hemorrhage was seen. The lumens of many collecting tubules were filled with cellular debris as a result of damage to the tubular epithelium in the cortex. The epithelium of the collecting tubules was somewhat swollen, but the changes here were not as marked as in the cortex.

Inclusion Bodies.—Sections from the urinary bladder, gastrointestinal tract, mucosa of the tongue, gall bladder, and brain were stained with hematoxylin and eosin or phloxine and examined for inclusion bodies. On one occasion, an inclusion-like body was seen in several cells of the molecular layer of the cerebellar cortex. Exam-

ination of similar sections from other infected pigs resulted in failure to repeat these findings.

Scrapings from the tongue and mucosa of the oral cavity of infected pigs were prepared and stained for inclusion bodies as described by Goss *et al.*[6] The findings in the epithelial cells of infected pigs were the same as those seen in normal pigs of the same age.

DISCUSSION

The gross and microscopic changes in transmissible gastroenteritis in pigs were largely limited to the gastrointestinal tract and kidneys. The causative agent seems to have an affinity for the epithelium of these organs since the greatest damage was found there. However, transmission studies indicated that other tissues of the body contained the causative agent. It may be that the causative agent was recently transported to these other tissues without causing noticeable pathologic changes.

The earliest change seen in the gastrointestinal epithelium of infected pigs was a mild congestion of the terminal blood vessels. This change seemed to indicate stimulation of the body defenses by some irritant substance. As the disease progressed, a more marked engorgement of the blood vessels of the mucosa, submucosa, and muscular layers developed. As the disease progressed, there was considerable desquamation of the surface epithelium, necrosis of epithelium in the crypts of Lieberkühn, and infiltration of the mucosa and submucosa by migratory cells. In many of the pigs that died when 2 to 4 days old, very little cellular infiltration of the gastrointestinal mucosa was observed; whereas increased numbers of lymphoid cells were seen in the stomach and intestinal tissues of pigs that lived longer. This was especially noticeable in the colon. The infiltration of neutrophils was limited largely to those areas where necrosis or necrobiotic changes occurred and was probably an attempt to segregate or remove the necrosed tissue. In pigs less than 2 weeks old, all of the above changes usually occurred within two to four days after the disease started and was usually followed by death. As the pigs became older, the percentage of recoveries increased.

The early miscroscopic changes observed in the kidneys of an infected pig were albuminous degeneration of the tubular epithelium of the cortex and congestion of both the cortex and medulla. In the later stages of the disease, there was considerable tubular epithelial desquamation, some areas of focal necrosis, and dilation of the tubules of the cortex. Grossly, large quantities of urates were seen in the renal pelvis.

SUMMARY

1) Transmissible gastroenteritis is a sporadic disease characterized by diarrhea, vomiting, dehydration, and high death losses in baby pigs.

2) On postmortem examination, there may be found, singly or in combination, gastritis, enteritis, degeneration of the kidneys, congestion of the mesenteric blood vessels, and an atony of the intestine with fluid contents.

3) The miscroscopic changes in the mucosa of the gastrointestinal tract varied from congestion of the terminal blood vessels to desquamation of epithelium, necrosis, and cellular infiltration.

4) In the kidney, albuminous degeneration, congestion of the blood vessels of the cortex and medulla, and desquamation of some tubular epithelium was the most common finding.

5) Inclusion bodies were not demonstrated with the techniques employed.

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The Bacteriologic Aspects of Experimental Brucellosis in Dogs Following Oral Exposure. I. Effects of Feeding Aborted Fetuses and Placentas to Adult Dogs

ERSKINE V. MORSE, D.V.M., Ph.D.; T. KOWALCZYK, DIP. VET., D.M.V.; B. A. BEACH, D.V.M.

Madison, Wisconsin

Literature

The bacteriologic and pathologic aspects of experimental canine brucellosis have received the attention of several investigators,[1,4,9,11,12] *Brucella abortus* served as the infecting agent in a minority of reports.[4,8,11,12] Exposure of dogs by the oral route has not resulted uniformly in infection.[4,11,12]

Van der Hoeden[12] succeeded in producing brucellosis in 10 of 15, 7-week-old puppies by feeding pure cultures of *Br. abortus*. Brucella were recovered from one or more of the following organs: spleen, liver, mesenteric lymph nodes, and tracheobronchial lymph nodes with considerable regularity at the time of autopsy during a 74-day postfeeding period. Brucella were isolated from the blood at one, eighteen, twenty-four, and thirty-two days after exposure. Agglutinins were demonstrable in the blood of 1 animal as early as the sixth day. Two puppies had titers of 1 : 3,200, the highest observed, eighteen days after feeding.

Feldman, Bollman, and Olson[4] fed 3 adult dogs cultures of *Br. abortus* mixed with raw meat. Unfortunately, 1 animal died of a volvulus, and at autopsy the material was not suitable for bacteriologic examination. An agglutinin titer was not demonstrated in this animal. Brucella were shown to be present at fourteen days in the blood of 1 dog, as demonstrated by guinea pig inoculation. The other 2 dogs were destroyed at ninety and one hundred eighty-five days, respectively. Their urine, spleens, and mesenteric lymph nodes, which were inoculated into guinea pigs, proved negative for Brucella. The peak blood titers of these 2 dogs were 1 : 200 and 1 : 800 at fifty, sixty, and 121 days, respectively.

These investigations were limited to the feeding of Brucella cultures. Hence, it seemed desirable, in order to simulate possible exposure as it may occur under farm conditions, to feed experimental dogs the aborted fetuses and placentas of Brucella-infected cattle. The following report deals with procedures and bacteriologic findings of such a simulated natural exposure experiment.

Materials and Methods

Fifteen adult dogs of mixed breeds were collected from the Madison, Wis., area. All were negative for Brucella on bacteriologic examination, and none of the serums showed a reaction at a dilution of 1 : 12.5 on the plate-agglutination test. The animals were fed a stock diet consisting of a dry dog meal and canned dog food.

The exposure material fed to the dogs consisted of aborted fetuses and placentas from cows infected with *Br. abortus* U. S. Department of Agriculture strain 2308. At the time of feeding, the infected tissues contained numerous viable Brucella.

The development of agglutinins was followed at approximately weekly intervals, using the plate test starting with the 1 : 12.5 dilution. However, animals which developed serum titers were later checked by the tube test. Bacteriologic examinations were made of blood, urine, and feces at weekly intervals. The femoral bone marrow and homogenates of liver, kidneys, spleen, lung, tonsil, and genital tract were examined similarly at autopsy.* Homogenates of the mesenteric lymph nodes and pooled samples consisting of bronchial-cervical, gastrohepatic-iliac, and popliteal-prescapular lymph nodes, respectively, were cultured in a like manner. The cervical lymph nodes included the submaxillary, retropharyngeal, and parotid nodes. Blood and urine obtained at autopsy were examined bacteriologically by placing 5- to 10-ml. quantities in rubber-stoppered bottles containing 50 ml. of heparin-treated tryptose broth. The cultures were incubated at 37 C. and subcultures were made on solid agar mediums on the fourth, seventh, and fourteenth days after inoculation. Blood, bone marrow, and other tissues were plated in duplicate or triplicate on Difco tryptose agar containing 0.00125 per cent sodium azide. Urine, other than that procured at autopsy, and fecal

From the Department of Veterinary Science, University of Wisconsin, Madison.

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*To accomplish this, the dogs were completely anesthetized by intravenous injection of nembutal ® and then exsanguinated by severing the femoral artery.

samples were cultured in duplicate on a special medium which inhibited most yeasts, molds, and bacteria other than Brucella. Unfortunately, the formula for this medium is classified government information and can not be released at this time. All plates were incubated at 37 C. for seven days, during which time daily examinations for the presence of Brucella colonies were made.

aborted material. Agglutination tests and bacteriologic examinations of blood and urine were made at two, five, and seven days and weekly thereafter. Only the catheterized urine samples from the females were cultured for Brucella. On the twenty-eighth day after feeding and periodically thereafter, fecal samples from the dogs

TABLE I—The Agglutinin Response of Dogs Fed Brucella-Infected Material

Dog 6	Day:	28	34	41	48	55	69	76	84	90	96	102	110	117	124	151
	Titer:	25	25	P50	P50	P50	50	50	25	P25	P50	25	64*	P128*	64*	128*
Dog 8	Day:	5	12	26	33	41	47	53	60	67	74	81	108	...	...	...
	Titer:	P25	P25	12.5	12.5	12.5	12.5	0	0	8*	4*	2*	4*	...	...	...
Dog 10	Day:	12	26	33	41	47	53	60	67	74	81	108	...	...	...	...
	Titer:	100	P100	100	100	100	50	50	P128*	P128*	P128*	P128*	...	...	...	...
Dog 15	Day:	14	22	29	35	42	49	56	53	97	105	116	...	...	...	...
	Titer:	12.5	P100	P100	100	100	P256*	128*	128*	64*	P64*	P64*	...	...	...	...

*Tube test results. Other figures represent plate-agglutination test findings. P = partial or incomplete agglutination.

Guinea pigs were inoculated intraperitoneally with the homogenized tissues of pooled lymph nodes, pooled viscera, and pooled genital tract, except lungs and tonsils. Approximately, 1 ml. of the homogenized material from dogs with titers and from pregnant females was inoculated into each of 3 guinea pigs. The guinea pigs were killed for autopsy and bacteriologic and serologic examination five weeks after the inoculations. Portions of all tissues which yielded Brucella on culture were sectioned for histologic study. The findings will be reported elsewhere.

The infecting organisms were identified as *Br. abortus* by the following criteria: 1) small, nonmotile, gram-negative rods or coccoid elements, (2) colonies typical of Brucella as viewed with a dissecting microscope with obliquely transmitted light, (3) ability to actively hydrolyze urea, (4) inability to ferment dextrose, (5) growth in the presence of basic fuchsin but not in the presence of thionin, (6) agglutination to the same end point as observed with the standard BAI tube antigen in high titer Brucella antiserum, and (7) ability to induce a serum-agglutination titer and typical brucellosis lesions in guinea pigs following intraperitoneal inoculation.

The experimental dogs were divided into four groups and handled as follows:

Group 1.—Group 1 consisted of 1 male dog (No. 3) and 5 female dogs (No. 1, 2, 4, 5, and 6). Two females were bred on about the seventeenth day and dog 6 became pregnant. The dogs were approximately 2 years old. The group was fed a single portion of

were examined for Brucella. Homogenized lymph nodes or viscera from the 2 female dogs which were bred were inoculated into guinea pigs.

Group 2.—Group 2 included 3 female dogs (No. 8, 9, and 10) and 1 male dog (No. 7). At the time of the single feeding of infected material, 2 of the female dogs (No. 8 and 9) were in the early stages of pregnancy, while the third, dog 10, was in estrus. Two females and the male were approximately 2 years old. Dog 8 was 12 years of age. Blood, urine, and feces were examined daily during the first six days after feeding and weekly thereafter. Guinea pigs were inoculated with homogenates of lymph nodes, viscera, and the genital tract of each female, respectively. The tissues of the male were not injected into guinea pigs.

Group 3.—Group 3 consisted of 3 male dogs (No. 11, 12, and 14) and 1 female dog (No. 13), ranging in age from 1 to 3 years. The group was fed a single meal of aborted tissues. Bacteriologic examinations were made of blood, urine, and feces at 2, 4, 8, 12, 18, 24, 36, and 48 hours after feeding. An animal from the group was killed at 12, 24, 36, and 48 hours, respectively. Bacteriologic examinations were made of the tissues at autopsy. Three guinea pigs were inoculated with pooled, homogenized lymph glands, and 3 with the pooled viscera from each dog, respectively.

Group 4.—Group 4 was represented by dog 15, a nonpregnant female, approximately 3 years old. She was fed the infected material a total of 26 times during a 51-day period. Agglutination tests and bacteriologic examinations of blood, urine, and feces were made weekly during the course of the 116-day experiment. At autopsy, the tissues were plated on bacteriologic mediums, and 3 guinea pigs received inoculations of pooled lymph gland material and 3 others were inoculated with viscera.

RESULTS

Group 1.—Dogs 1 and 2, both of which were nonpregnant females, were destroyed fifteen days after feeding. Neither had shown an agglutinin response. Brucella were not recovered from the blood or urine. The tissues examined at autopsy were bacteriologically negative.

Dog 3, a male, and dog 4, a nonpregnant female, were killed for autopsy on the forty-first day of the experiment. Neither animal had a blood titer. A Brucella-like organism was isolated from the blood of dog 3 at forty-eight hours. Unfortunately, the isolate was discarded before extensive confirmatory tests were performed. The colonies were, however, typical of *Br. abortus*, and the organisms were agglutinated to a dilution of 1 : 200 by high titer Brucella antiserum. Other organisms isolated during the course of this study which reacted similarly proved to be *Br. abortus*. None of the other bacteriologic examinations made of this dog were positive. Guinea pigs were not inoculated.

Brucella were recovered from the bronchial and cervical lymph nodes of dog 4. Other attempts at isolations were negative and guinea pig inoculations were not made.

Dog 5 was a female which had been bred on the twenty-eighth day following the feeding. She was destroyed at eighty-four days and, at this time, was in pseudopregnancy. An agglutinin response was not observed. Cultures for Brucella made from blood, urine, and feces were negative. Brucella were recovered from the mesenteric lymph nodes. Guinea pig inoculations were negative.

Dog 6 whelped a normal litter of 7 young on the seventy-ninth day after feeding. A postpartum vaginal discharge which persisted for three days was negative for Brucella. No elevation in temperature was observed during the four weeks following parturition and the animal appeared in good health. An agglutinin titer of 1 : 25 was present shortly after estrus at twenty-eight days. At 1 month of age, all the young were negative on the tube-agglutination test at a 1 : 2 serum dilution. Five days following parturition, the titer of the bitch dropped but, as indicated in table 1, rose again after the young were weaned. This female was killed 151 days after feeding the infective material. Brucella were isolated from the pooled bronchial and cervical lymph nodes. Blood, urine, and feces remained negative on culture for Brucella.

TABLE 2—Summary of Positive Bacteriologic Examinations of the Dogs' Tissues, Urine, and Feces

Dog (No.)	Specimen taken at	Blood	Urine	Feces	Viscera	Lymph node group		
						Bronchial-cervical	Mesenteric	Gastro-hepatic-iliac
14	2 hours	—	—	+	..	..	..	..
14	4 hours	—	+	—	..	..	..	..
12	8 hours	—	+	—	..	..	..	..
11	12 hours	—	—	—	+	—	—	—
3	48 hours	+	..	..	..	..	..	..
4	41 days	—	—	—	—	+	—	—
9	41 days	—	—	—	—	+	—	—
5	84 days	—	—	—	—	—	+	—
8	108 days	—	—	—	—	+	—	—
10	108 days	—	—	—	—	+	+	+
6	151 days	—	—	—	—	+	—	—
15	116 days	—	—	—	—	+	—	—

Guinea pigs which received the homogenized lymph nodes, viscera, and genital tract, respectively, were negative at autopsy.

Group 2.—Dog 7 was a male. Bacteriologic examinations of blood, urine, and feces, which were made daily for six days postfeeding, were all negative. Agglutination tests were negative. Autopsy was performed on the sixth day. Brucella were not recovered from the tissues. Guinea pig inoculations were not made.

Dog 8 had been pregnant about two weeks when fed the aborted material. On the fourth day after the feeding, a bloody, mucopurulent vaginal discharge was observed. The discharge persisted for eight days only to reappear at thirty-five days and to persist for about twenty days. The dog remained in good health, with a normal temperature, and Brucella were not isolated from the vaginal discharge. A normal male pup was whelped on the fiftieth day of the experiment. At 6 weeks of age, tests for Brucella agglutinins were negative. The female dog's agglutinin titer was complete at 1 : 12.5 and partial at 1 : 25 five days after feeding and remained positive at 1 :12.5 until whelping. At that time, an agglutinin titer was lacking, but a peak of partial at 1 : 25 was regained approximately twenty-four days after parturition. The results are summarized in table 1. No Brucella were isolated from the blood, urine, or feces prior to necropsy on the 108th day but, at that time, the pooled bronchial and cervical lymph nodes yielded positive cultures. Guinea pigs inoculated with either lymph nodes, viscera, or genital tract were negative.

Dog 9 showed signs of early pregnancy when fed the infective material. It was felt the bitch aborted and ate the fetuses and afterbirth at about the twenty-sixth day of the experiment. At this time, there was swelling of the vulva to about twice normal size and a profuse mucopurulent vaginal discharge. The discharge persisted for twenty-five days. Brucella were not cultured from the discharges. The temperature remained normal, there was considerable loss of weight, and the coat became dull and rough. No Brucella were recovered from the urine, blood, or feces at any time. Agglutinins were not detected. At autopsy, on the forty-first day after feeding, Brucella were isolated from the bronchial-cervical lymph node pool. The uterine horns were larger than normal and numerous corpora lutes were present. Three guinea pigs which were inoculated with genital tract material and 3 others which received viscera and lymph nodes were negative.

Dog 10 was in estrus the third day after the aborted material was fed. This animal was kept with a male dog during the period and appeared to be in excellent health and gained in weight. About twenty-one days after breeding, a profuse, thick, bloody, mucopurulent vaginal discharge was observed. Mucoid material admixed with blood and resembling fetal membranes was found under her cage. Cultures for Brucella, and guinea pig inoculations, were negative. The guinea pigs died in about forty-eight hours, apparently from contaminating organisms, and Brucella were not isolated from them. Vaginal discharge continued intermittently during the following forty-one days. Frequent attempts to isolate Brucella from them failed. The dog lost weight and the coat became dull. Temperature readings ranged from normal to a peak of 105.6 F., the latter at seven days after the suspected abortion. Twelve days after feeding, the agglutinin titer was, as indicated in table 1, 1 : 100 and continued at this level during most of the experiment. Attempts to culture Brucella from the blood, urine, or feces were negative throughout the course of the investigation. Autopsy was performed at 108 days. Brucella were recovered from the enlarged mesenteric lymph nodes and the bronchial-cervical and gastrohepatic-iliac lymph nodes, respectively. The spleen was approximately twice its normal size. The genital tract appeared normal. Guinea pigs which were inoculated with material from either the viscera, lymph nodes, or genital tract were negative for Brucella.

Group 3.—Dog 11, a male, was destroyed for necropsy twelve hours after feeding the aborted material. All cultures of the tissues were negative. However, guinea pigs inoculated with the pooled viscera sample, which was comprised of liver, spleen, kidney, and testicle, showed the typical lesions of brucellosis. Blood titers were observed, and Brucella were recovered from their tissues. Dogs 12, 13, and 14 were bacteriologically negative on autopsy at 24, 36, and 48 hours, respectively.

Urine obtained from dog 12 on the eighth hour after the feeding contained Brucella. The two-hour fecal sample as well as the four-hour urine specimen of dog 14 were bacteriologically positive. Except for these instances, Brucella were not isolated from blood, urine, and fecal samples obtained at 2, 4, 8, 12, 18, 24, 36, and 48 hours.

male, was fed the aborted material repeat-

Group 4.—Dog 15, a nonpregnant female, was fed the aborted material repeatedly. Blood agglutinins were detected on the fourteenth day. A peak titer of partial at 1 : 256, as indicated in table 1, was

reached at fifty-six days. On the twenty-second day, a mucopurulent vaginal discharge was observed. It continued for the following thirty-five days, but Brucella were not recovered from the discharge. The temperature remained normal and the dog appeared in good health. All cultures of the blood, urine, or feces were negative throughout the investigation. Postmortem examination was made on the 116th day after the feeding was commenced. Brucella were recovered from the pooled bronchial and cervical lymph nodes. Guinea pigs inoculated with the homogenized lymph glands from this dog were infected with *Br. abortus* when examined at autopsy.

The results of the bacteriologic examinations on the 15 dogs are summarized in table 2.

DISCUSSION

These studies indicate that dogs may harbor *Br. abortus* in one or more lymph nodes for long periods following a single infective meal of aborted material. The pathogen was only rarely eliminated in the urine and feces. It would, therefore, seem that dogs seldom, if ever, void sufficiently large numbers of the organism to produce infection in other animals. The infective discharges of an aborting bitch may, however, represent substantial hazards at times.[2]

A pregnant dog may abort after eating the fetuses or placentas of Brucella-infected cattle. The female which is in estrus or pregnancy is apparently more subject to infection than is the female with a dormant genital tract or the male dog.

Dogs which had agglutinin titers harbored Brucella in one or more lymph nodes. Dogs which had no demonstrable agglutinins in their blood still harbored Brucella for long periods after a single meal of infective material.

Brucella abortus only occasionally caused a bacteriemia after its ingestion, as indicated by the fact that we were able to isolate Brucella from the blood of only 1 dog. Feldman *et al.*[4] had a similar experience. Van der Hoeden[11] recovered Brucella from the blood of 4 of 15 dogs after oral exposure.

Dogs may show a substantial response to contact and infection with *Br. abortus*. Abortion, a chronic vaginal discharge, and the development of an agglutinin titer were associated with feeding Brucella-infected tissues, although little diagnostic significance could be attached to the symptoms. Some animals lost weight following exposure, had a dull, dry coat, and an undulating type of fever was demonstrated. Lameness has been noted as being significant in male dogs by Cardona,[1] Davis,[3] and Planz and Huddleson.[18]

SUMMARY

Dogs were found to harbor virulent Brucella in one or more lymph nodes for a period of 151 days following a single meal of aborted fetal and placental materials from cows infected with *Brucella abortus* U. S. Department of Agriculture strain 2308.

Shortly after feeding, Brucella were recovered from the urine on two occasions and once from the feces. Bacteriemia was shown to exist in only 1 dog at forty-eight hours after exposure, thus indicating it is rare.

Agglutinins did not appear in the serums of all infected dogs, but all animals which developed titers were found to be infected at autopsy. Agglutinins were demonstrable over a period of 151 days.

Two pregnant animals appeared to have aborted following the single oral exposure. Two infected females whelped normal, healthy litters at term.

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Effect of a Vitamin B_{12} Concentrate and Liver Meal on the Histopathology of Chicks Fed an All-Plant Protein Ration

M. J. SWENSON, D.V.M., M.S., Ph.D.

Ames, Iowa

IN PREVIOUS papers,[1,2] the effect of a vitamin B_{12} concentrate and liver meal on growth, feed efficiency, and hematology of chicks fed an all-plant protein ration was reported. Significant growth responses were obtained in the female chicks by replacing 3 per cent of the soybean meal in the basal ration with 3 per cent liver meal and also by replacing 0.5 per cent of the soybean meal in the basal ration with 0.5 per cent animal protein factor (APF) supplement No. 3 of Merck and Company. The APF ration contained 62.5 μg. of vitamin B_{12} per pound by the *Lactobacillus lactis* Dorner method of assay. Under the same conditions, growth responses of the male chicks were not significant statistically except at 2 weeks of age. Environmental effects were present in the weight gains of male chicks, and also the standard errors of the mean weight gains of the male chicks were larger than those of the female chicks. These findings indicate that the female chick should be a better assay animal for vitamin B_{12} than the male chick. The feed efficiency of the chicks fed the liver meal ration was the best throughout the experiment. There was no significant difference in feed efficiency among the chicks fed the basal and APF rations. Statistically, a significant difference was found between the hemoglobin levels of the female chicks fed the basal and liver meal rations, while no significant difference existed between the hemoglobin levels of chicks fed the basal and APF rations and also between those fed the liver meal and APF rations. The hemoglobin levels of the male chicks fed the three rations were not significantly different. The packed erythrocyte volume of the female chicks fed the basal and liver meal rations approached the 5 per cent level of significance. Treatment effects on erythrocyte counts, total leucocyte counts, differential counts, sedimentation rates, plasma volume, and packed leucocytes and thrombocytes were not significant. Age (21 to 37 days) produced a highly significant difference in the hemoglobin levels of chicks and a significant difference in the eosinophil counts, whereas the remaining blood determinations had not changed significantly.

The author is now in the Department of Physiology, School of Veterinary Medicine, Kansas State College, Manhattan.

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MATERIALS AND METHODS

Ninety New Hampshire chicks, hatched from eggs produced by hens fed an all-plant protein ration for five months, were assigned at random to nine groups with 10 chicks in each group. A description of the rations and experimental procedure has been described in a previous paper.[1] The ingredients of the rations are presented in table 1.

At the end of twelve weeks, 18 chicks were killed for gross and microscopic study of tissues. The smallest, largest, and medium-sized chicks from the three groups receiving the same treatment were killed. In designing the experiment, it was decided to select tissues from these chicks for histopathologic study. Also, 2 chicks from each group and an equal number of males and females were scheduled to be killed for tissue study. Thus, the first chicks assigned at random in each group meeting these requirements were killed, with the exception of the 6 male chicks which developed perosis.[1] The chicks were placed in a scale funnel with their heads down. They were killed and bled by decapitation 1 in. posterior to the occipito-atloid joint.

After the bleeding was completed, a portion of sciatic nerve was removed and fixed in 10 per cent formol-saline solution for twenty-four hours. Longitudinal sections, 10μ in thickness, were then made of the nerve with a freezing microtome. The sections were floated in water, placed on a clean slide, mounted in glycerin, covered with a clean cover glass, and examined immediately between crossed prisms of a polarizing microscope for myelin sheath degeneration. Photomicrographs were taken of the nerves at the point of greatest birefringence.

Various tissues were stained with hematoxylin and triosin and by the Marchi method.[5] The tissues were removed immediately after killing and placed in the fixative. The tissues consisted of small portions of brain, spinal cord, sciatic nerve, skeletal muscle, heart, liver, kidney, spleen, pancreas, gizzard, proventriculus, small intestine, cecum, rectum, testis or ovary, thyroid, thymus, and lung. The tissues stained by the Marchi method were embedded and sectioned 20μ in thickness. Tissues stained with hematoxylin and triosin were fixed in Zenker's solution, embedded, and sectioned 6μ in thickness.

TABLE 1—Ingredients of Rations

Ingredients	Rations		
	Basal (%)	Liver meal (%)	APF (%)
Yellow corn, ground	35.0	35.0	35.0
Oats, ground	5.0	5.0	5.0
Wheat bran	10.0	10.0	10.0
Wheat midds	10.0	10.0	10.0
Corn gluten meal	10.0	10.0	10.0
Soybean meal, 41%	16.5	12.5	14.0
Alfalfa meal, dehyd.	5.0	5.0	5.0
Steamed bone meal	1.0	1.0	1.0
Pulverized limestone	1.5	1.5	1.5
Iodized salt	0.5	0.5	0.5
Cod liver oil	0.5	0.5	0.5
Choline premix*	1.5	1.5	1.5
Riboflavin premix**	1.0	1.0	1.0
Trace mineral premix†	2.5	2.5	2.5
Liver and glandular meal	...	3.0	...
APF premix‡	...	...	2.5
Total	100.0	100.0	100.0

*Choline premix contained 5.0 per cent choline. **Riboflavin premix contained 0.022 per cent riboflavin. †Trace mineral premix contained 3.0 per cent "CCC" trace mineral premix for poultry. ‡APF premix contained 20.0 per cent APF supplement No. 3 of Merck and Company.

RESULTS

Various degrees of degeneration were observed in the myelin sheaths of the sciatic nerves of the 18 chicks between crossed prisms of the polarizing microscope. The photomicrographs in fig. 1 represent the average amount of myelin sheath degeneration from the chicks fed the three rations. The sciatic nerves of all the chicks showed some myelin sheath degeneration. The treatments (rations) did not cause an appreciable difference in the degree of myelin sheath degeneration. In studying myelin sheaths with a polarizing microscope, the swelling of myelin sheaths indicates a degenerative change which eventually may lead to severe degeneration. The position of the longitudinal section in relation to the axis cylinder of the nerve fiber may alter the general appearance, especially in the width of the anisotropic material (white) as shown in fig. 1. The nodes of Ranvier and the incisures of Schmidt-Lantermann in the myelin sheaths appear isotropic (black) in the photomicrographs and must not be confused with the degenerative areas.

The results of the Marchi method of staining tissues are summarized in table 2. These observations are not necessarily

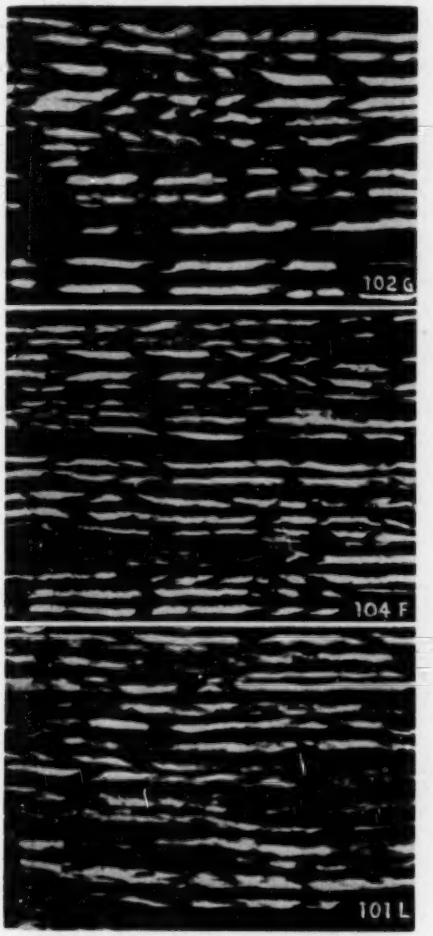

Fig. 1—Photomicrographs taken between crossed prisms of a polarizing microscope at the point of greatest birefringence of sciatic nerves from chick 102G fed the basal ration, chick 104F fed the liver meal ration, and chick 101L fed the APF ration. x 500.

pathologic because osmic acid stains fat wherever it is present. The observations recorded for the spleen, pancreas, proventriculus, small intestine, cecum, rectum, and thymus are considered normal. The alterations (one plus and two plus) observed in the brain medulla, cerebellum, spinal cord, sciatic nerve, heart, and liver are considered pathologic. Those changes recorded as traces are considered either normal or of minor pathologic importance due to the inadequacy of the Marchi staining method.[4]

The fatty change in the liver was most prevalent in chicks fed the APF ration (table 2), followed in order by the liver meal and basal rations. Figure 2 reveals the presence of the fatty change in the hepatic cells of the liver from chick 101L fed the APF ration. This change was not limited to the area around the central vein, as the photomicrograph may suggest, but was uniformly present throughout the liver lobules.

Figure 3 demonstrates the normal distribution of fat in the yolk follicles of the ovary. Numerous small follicles can be seen near the margins of the photomicrograph. Fat was also observed in the interstitial cells of the nine testes examined microscopically. Minute fat droplets were present in almost every cell of the glandular portion of the pancreas and proventriculus of the chicks (table 2). These droplets were small and uniform in size. The spleen, thymus, and lymphoid tissue present in the wall of the small intestine, cecums, and rectum often contained a small amount of material which stained black with osmic acid.

Portions of tissue corresponding to those taken from chicks for the Marchi method of staining were stained with hematoxylin and triosin. Pathologic changes were observed in the heart, liver, and kidney. The changes in the heart will be discussed later. A fatty change, indicated by the appearance of clear vacuoles in the hepatic cells, was noted in the livers of chicks 109E, 102L, and 101L showing a two plus change by the Marchi method (table 2). Part of the livers with a one plus or trace showed some evidence of vacuolization in the hepatic cells. These vacuoles were smaller and less numerous than those with a two plus change. Albuminous degeneration was present in the tubule cells of the kidneys from all the chicks except 101C fed the APF ration and 101J and 103L fed the basal ration. The nuclei of the affected tubule cells were pyknotic. An increased cellularity was observed in the center of the capillary tufts of the glomeruli in the kidneys of all the chicks as compared with mammalian kidney. Hyalinized areas, 10 to 50μ in diameter, were present in the medulla of the thymus. These areas are distinct from Hassall's corpuscles and yet they appear in the same general location.

TABLE 2—Results of the Marchi Staining Method

Ration	Chick (No.)	Sex	Weight gain (Gm.)	Brain medulla	Cerebellum	Spinal cord	Sciatic nerve	Skeletal muscle	Heart	Liver	Kidney	Spleen	Pancreas	Proventriculus	Gizzard	Small intestine	Cecum	Rectum	Testis*	Ovary*	Thyroid	Thymus	Lung
Basal	109G	F	791	T	T	T	T	—	—	+	—	—	T	T	—	T	—	T	..	—	—	T	—
	101J	F	1,293	T	T	T	T	—	T	T	T	—	T	T	—	T	T	T	..	—	—	T	—
	103L	F	1,449	T	T	+	+	—	T	—	T	—	T	T	—	T	—	T	..	—	—	T	—
	104E	M	1,549	+	+	+	+	—	T	T	T	T	T	T	—	T	—	—	..	..	—	T	—
	102G	M	1,716	+	+	+	T	—	T	T	T	T	T	—	—	—	—	—	..	..	—	T	—
	101K	M	1,988	+	+	+	+	—	T	T	T	—	T	T	—	—	T	T	..	..	—	—	—
Liver meal	102J	F	1,272	T	T	+	T	—	T	T	—	—	T	T	—	—	T	T	..	—	—	T	—
	101F	F	1,311	+	+	+	+	—	T	T	T	—	T	T	—	T	T	—	..	—	—	T	—
	109E	F	1,603	T	T	T	T	—	T	++	T	—	T	T	—	T	—	T	..	—	—	—	—
	105E	M	1,800	+	+	+	+	—	T	+	T	—	T	T	T	T	—	—	..	..	—	T	—
	102F	M	1,996	+	+	+	+	—	—	T	T	—	T	T	—	T	T	—	..	..	—	—	—
	104F	M	2,055	+	+	+	+	—	+	T	T	—	T	T	—	T	T	T	—	..	—	T	—
APF	108J	F	1,195	T	T	T	T	—	—	+	T	—	T	T	—	T	—	T	..	—	—	T	—
	102L	F	1,401	+	+	+	+	—	+	++	T	T	T	T	—	T	T	T	..	—	—	T	—
	103F	M	1,454	+	+	++	+	—	T	+	T	—	T	—	—	T	—	—	..	..	—	T	—
	101L	F	1,546	T	T	T	T	—	—	++	—	—	T	T	—	—	—	—	..	..	—	T	—
	101C	M	1,723	T	T	T	T	—	T	T	T	—	T	T	—	T	T	T	..	..	—	T	—
	106I	M	2,065	+	+	+	+	—	+	T	T	—	T	T	—	T	T	—	—	..	—	+	—

—signifies no fatty change or deposition; T signifies trace, only enough for identification; + signifies moderate amount, less than 25 per cent of area or number affected; ++ signifies marked fatty change, 25 to 50 per cent of area or number affected; * not including yolk follicles of the ovary and interstitial cells of the testis.

The macroscopic changes observed in the tissues and organs of the 18 chicks autopsied at the close of the experiment were confined primarily to the hearts and gizzards. A majority of the chicks' hearts had diffuse areas of grayish discoloration in

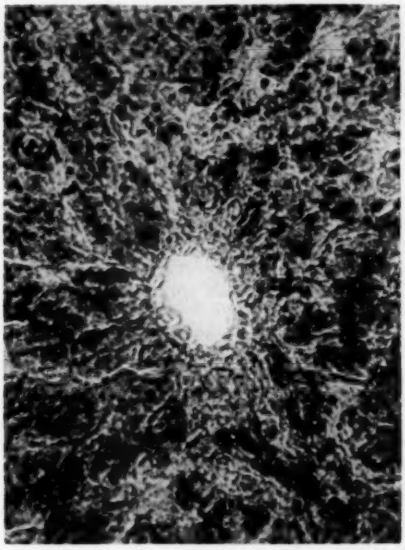

Fig. 2—Liver of chick 101L fed the APF ration showing a fatty change around a central vein. Marchi stain. x 500.

the muscle adjacent to the epicardium. Microscopic examination of sections made of the heart muscle revealed an areolar type of connective tissue joining the epicardium to the normal heart muscle fibers.

Eight gizzards from the 18 chicks autopsied showed erosions varying from 1 to 8 mm. in diameter. These areas were localized in all cases. Four of the affected chicks had been fed the basal ration, 3 chicks the liver meal ration, and 1 the APF ration. Microscopic examination of sections made of the affected gizzards showed that the erosions were confined to the keratinized epithelial layer.

The cecums of the 18 chicks appeared to be extremely long. Those of the smallest chicks were 7 in. long, while those of the largest chicks measured a maximum of 11 in. The cecal contents appeared normal. Microscopic examination of the cecal and rectal contents failed to demonstrate the presence of internal parasites.

Chick 106J fed the liver meal ration had lost weight the last two weeks of the experiment. The chick had become thin and emaciated. On autopsy, a mass of tissue, 4 by 10 by 12 cm., was found in the abdominal cavity, dorsal to the intestine, largely to the right of the median plane, and replacing the anterior lobe of the right kidney. This tissue was gray, solid, easily torn by applying pressure, cut easily, and gray on the cut surface. Figure 4 shows that this mass of tissue was an embryonal nephroma.

DISCUSSION

The leg weakness observed in chicks fed the basal and APF rations[1] was not correlated with the myelin sheath degeneration, because chicks fed the liver meal ration showed as much myelin sheath degeneration as those fed the other rations. The results from the Marchi method of staining checked closely with the observations made

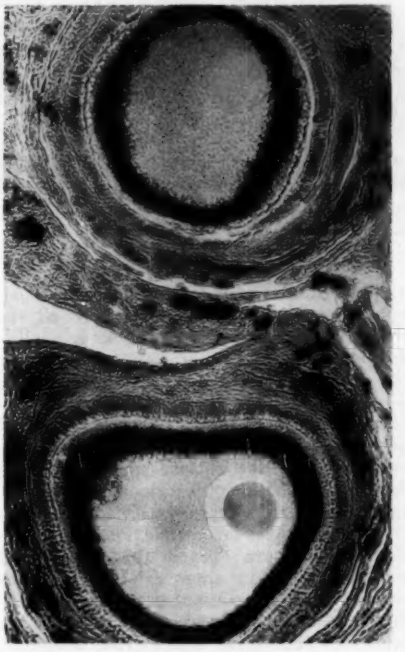

Fig. 3—Ovary of chick 103L showing the distribution of fat in the yolk follicles. Marchi stain. x 500.

with the polarizing microscope between crossed prisms at the point of greatest birefringence.

The presence of the fatty change observed in the livers (table 2 and fig. 2) and also the degenerative changes of the

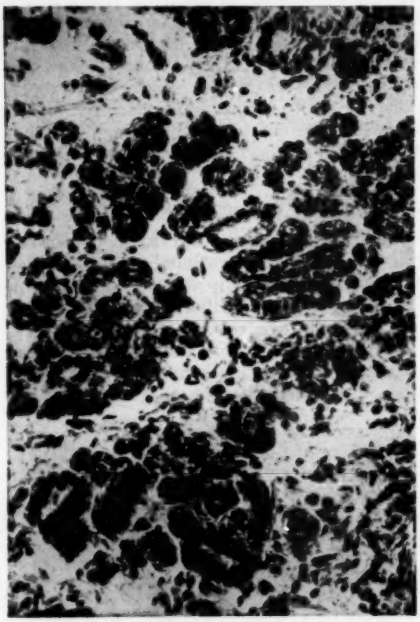

Fig. 4—Embryonal nephroma obtained from the abdominal cavity of chick 106J. Hematoxylin and triosin stain. x 300.

kidney tubules (pyknotic nuclei and albuminous degeneration) of nearly all the chicks listed in table 2 suggest a choline deficiency. These alterations conform to the described pathologic changes in the liver and kidney occurring in choline deficiency of mammals.[5] The occurrence of perosis in 6 of the 90 chicks[1] is further evidence that the rations may have been deficient in choline. The amount of choline in the rations, however, exceeded the quantity recommended by the National Research Council.[6] Kilborn[7] found that ducks and pigeons failed to show a significant increase in the fat content of livers by feeding a choline-free, high fat diet. Kilborn concluded that birds differ from mammals in fat metabolism. Fasting, fasting plus anterior pituitary injections, or choline-deficient diets did not cause a significant increase in the fat content of livers of chickens, whereas such treatments are known to cause a significant increase in the liver fat of mammals.

The minute black droplets observed in the enzyme-secreting cells of the pancreas and proventriculus and in the lymphoid tissue stained by the Marchi method (table 2) are considered normal. The interval between feeding and killing of the chicks may have contributed to the slight variation in the results from the fat stain. The chicks were self-fed, having feed in front of them at all times. It is believed that the hyalinized areas in the medulla of the thymus may eventually become the centers of Hassall's corpuscles because the hyalinized areas are in the medulla of the thymus and Hassall's corpuscles have hyalinized centers.

The feeding of an all-plant protein ration to the hens may have accounted for the incidence of the gizzard erosions in 8 of the 18 chicks autopsied. Mushett and Ott[8] found fewer and less severe gizzard erosions in chicks fed rations containing vitamin B_{12} than chicks fed the same rations without vitamin B_{12}. The lower incidence of gizzard erosions in chicks fed the APF ration in this investigation agrees with Mushett and Ott.

Sisson and Grossman[9] state that the cecums of the chicken are about 7 in. long, whereas those from the autopsied chicks measured 7 to 11 in. Lippincott and Card[10] state that the cecums of the chicken are from 4 to 6 in. long. Perhaps those observed as being longer than usual in this investigation were due to heredity, type of ration, confinement of chicks, or some unknown factor. The fiber content of the rations was not excessive, being less than 6 per cent. The abundance of connective tissue between the epicardium and the normal heart muscle fibers is probably the result of the needle puncturing the heart in the bleeding process.

SUMMARY AND CONCLUSIONS

1) The leg weakness of chicks fed the basal and animal protein factor (APF) rations was not associated with myelin sheath degeneration.

2) Treatments (rations) produced no appreciable change in the degree of myelin

sheath degeneration in sciatic nerves of chicks as observed between crossed prisms of a polarizing microscope at the point of greatest birefringence and by the Marchi method of staining.

3) The Marchi method of staining and the hematoxylin-triosin stain revealed a fatty change in the liver, the greatest change appearing in the livers of chicks fed the APF ration followed in order by those fed the liver meal and basal rations.

4) Pyknotic nuclei and albuminous degeneration were observed in the tubule cells of the kidneys stained with hematoxylin and triosin.

5) The kidney and liver changes with the occurrence of 6 cases of perosis in male chicks suggest a choline deficiency.

6) Eight gizzards from the 18 chicks autopsied had erosions in the keratinized portion of the epithelium, while the glands in the tunica propria of the mucous membrane appeared normal microscopically.

7) The cecums of the chicks were unusually long, varying from 7 to 11 in., but showed no pathologic changes microscopically.

8) Grossly, a majority of the hearts showed areas of grayish discoloration in the muscle under the epicardium which on microscopic examination revealed an areolar type of connective tissue joining the epicardium to the normal muscle fibers. This condition was attributed to the needle puncturing the heart in obtaining blood samples.

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Concentration of the Virus of Infectious Sinusitis of Turkeys with Methyl Alcohol

J. E. PRIER, D.V.M., M.S.

Urbana, Illinois

THE ETIOLOGIC agent of infectious sinusitis of turkeys has been reported to be either a virus or a Rickettsia.[3, 4, 5, 6] The agent has been demonstrated in the lungs, air sacs, and sinus exudate of infected birds. The usual copious sinus exudate of the disease is a convenient source of virulent material. Since the infraorbital sinuses of fowl are bounded laterally by skin, a needle can easily be introduced into the cavity to withdraw the contents. The sinus exudate is thick and tenacious and is not readily miscible with water. This factor makes it difficult to obtain a uniform suspension of infective material.

A method has been developed[1] for the purification and concentration of influenza and other viruses using methyl alcohol at low temperatures. A similar method[2] has been used for the purification of the virus of infectious rabbit papillomatosis. The present paper describes the use of methyl alcohol for concentration of the agent of infectious sinusitis of turkeys.

TABLE I—Response of Experimental Turkeys to Intrasinusoidal Inoculation with Methyl Alcohol-Precipitated Infectious Sinusitis Virus

Age of turkeys*	Inoculum	Dilution	No. birds dead or showing symptoms	No. days after inoculation
2 months	Alcohol-precipitated virus suspended in phosphate buffer**	u.d.+	1 dead 2 nasal discharge	dead—6 symptoms—8
2 months	Alcohol-precipitated virus suspended in phosphate buffer**	10^{-1}	2 dead 1 nasal discharge	dead—7 symptoms—8
2 months	Alcohol-precipitated virus suspended in phosphate buffer**	10^{-2}	1 dead	6
2 months	Alcohol-precipitated virus suspended in phosphate buffer**	10^{-3}	1 swollen sinus	12
2 months	Alcohol-precipitated virus suspended in phosphate buffer**	10^{-4}	1 nasal discharge	8
2 months	Alcohol-precipitated virus suspended in phosphate buffer**	10^{-5}	1 dead	6
2 months	Nasal exudate from infected turkeys	10^{-1}	3 swollen sinuses	10
2 months	Nasal exudate from infected turkeys	10^{-2}	1 dead 2 swollen sinuses	dead—6 symptoms—10 and 14
2 months	Nasal exudate from infected turkeys	10^{-3}	1 swollen sinus	14
2 months	Nasal exudate from infected turkeys	10^{-4}	none	..
4 months	Alcohol-precipitated virus suspended in phosphate buffer**	u.d.	3 swollen sinuses	12
4 months	5 ml. supernatant from precipitate material	u.d.	none	..

*Three birds used in each group. **pH 7.6. +Undiluted.

From the College of Veterinary Medicine and Agricultural Experiment Station, University of Illinois, Urbana.

The technical assistance of Mr. Gerald Dart is acknowledged.

At present, the author is head of the Department of Veterinary Science and Bacteriology, University of Wyoming, Laramie.

MATERIALS AND METHODS

The sinus exudate of experimentally infected birds was used as a source of virus. The infection was produced by intrasinusoidal inoculation of 1 ml. of sinus exudate from naturally

infected turkeys. Fifteen milliliters of the exudate was mixed with 30 ml. of physiologic saline and ground in a Waring blendor. The relatively homogeneous suspension (pH 7.6) was then centrifuged for thirty minutes at 2,000 revolutions per minute in an International #2 centrifuge to remove the larger particles.

The supernatant (pH 7.4) was decanted, chilled to 1 C. in a dry ice bath, and 11 ml. of absolute methyl alcohol held at –40 C. in a dry ice-methyl cellusolve bath was added to it, drop by drop, with constant agitation. The mixture was held at 1 C. for one hour, and then centrifuged in the International #2 centrifuge for thirty minutes at 5 C.

The resulting supernatant was removed and the precipitate resuspended in 10 ml. of a phosphate buffer solution (pH 7.6). The suspension was allowed to elute at 5 C. for twelve hours before animal inoculation.

The turkeys used in these experiments were of two age groups: 2 and 4 months. All birds were of the Broad Breasted Bronze breed. All inoculations were made into the left infraorbital sinus. Infection was determined by visible swelling at the site of inoculation or the presence of a nasal discharge. The strain of the virus (Illinois K) used in these studies has been shown consistently to produce visible swelling nine to fourteen days after inoculation. Inoculated birds were observed for the twenty-day period immediately following inoculation.

RESULTS

The data are summarized in table 1. One group of 2-month-old birds was used to titrate the resuspended methyl alcohol precipitate. Decimal dilutions through 10^{-5} were made and 3 turkeys inoculated with each dilution, using 0.5 ml. in the left sinus. One bird given undiluted material died six days after inoculation, and 2 birds in the 10^{-1} group died seven days after inoculation. All other birds in these two groups showed a nasal discharge eight days after injection of the diluted precipitate suspension. One bird receiving the 10^{-2} and 1 receiving the 10^{-5} dilutions died six days after inoculation. One bird in the 10^{-4} group showed a nasal discharge eight days after injection, and 1 bird in the 10^{-3} group showed a slight swelling of the sinus twelve days after injection. The birds were observed for twenty-five days following inoculation.

A similar group of 2-month-old turkeys was inoculated with dilutions of a suspension of the sinus exudate. Dilutions of 10^{-1} through 10^{-4} were used. One bird in the 10^{-2} group died six days after inoculation, and all remaining birds in the 10^{-1} and 10^{-2} groups showed swelling of the infraorbital sinus by the fourteenth day after inoculation. One bird in the 10^{-3} group also showed symptoms of sinusitis.

A similarly prepared methanol precipitate was used to inoculate 3, 4-month-old turkeys—1 ml. into the left infraorbital sinus of each bird. Three control birds were inoculated with the alcohol supernatant obtained following centrifugation of the precipitated material. All birds inoculated with the resuspended precipitate showed swelling of the left infraorbital sinus twelve days later. Those inoculated with the supernatant remained negative.

SUMMARY

The virus of infectious sinusitis of turkeys was precipitated by methyl alcohol at low temperatures. There was, apparently, nearly 100 per cent recovery of the organism in the resuspended precipitate as determined by inoculation of experimental turkeys. There apparently resulted from the infection some increase in lethal characteristics of the organism for young turkeys.

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Susceptibility to Newcastle Disease Infection of Chickens from Hens Immunized with Live Virus Vaccines

E. R. DOLL, M.A., D.V.M.; WILLIAM H. McCOLLUM, M.S.;
M. ELIZABETH WALLACE, M.S.

Lexington, Kentucky

THE STUDY reported here was undertaken to obtain information on the susceptibility to Newcastle disease (ND) infection of chickens hatched from actively immunized hens and to obtain further information on the activity of Newcastle disease virus (NDV) in groups of chickens with congenital passive antibody.

LITERATURE

The presence of a yolk-conferred passive immunity in the progeny of hens recovered from or immunized against ND was described by Brandly, Moses, and Jungherr.[1] Their experiments indicated that the passive protection against intramuscular inoculation with live NDV could persist for about four weeks. Also, the passive immunity interfered with immunization following intramuscular inoculation of live NDV or active immunization with formalin-inactivated vaccine. Levine and Fabricant[2] reported natural occurrence of ND infection among 2-week-old chickens hatched from vaccinated hens. In experimental studies, they found that intranasal inoculation of chickens hatched from vaccinated and recovered hens caused respiratory infection of every chicken regardless of serum-neutralization (SN) or hemagglutination-inhibition (HI) titers of the serum. Infection of young chickens hatched from immune hens and the occurrence of an actively immunizing reaction resulting from the infection was reported by Doll, McCollum, and Wallace.[3]

PROCEDURES

The HI test used in this study was adapted from the methods of Beach[4] and Brandly *et al.*[5] It employed 0.25-cc. twofold serial dilutions of fresh chicken serum in physiologic saline, 10 hemagglutination (HA) units of NDV strain Ky-127, and 0.5 cc. of a 0.5 per cent suspension of chicken red blood cells. The serum-virus mixtures were incubated ten minutes at room temperature before adding the red cells, and the tests were read forty-five minutes after addition of the red cell suspension using complete inhibition of agglutination as the end point. Titers were expressed as the highest serum dilution causing complete inhibition of agglutination. The HA unit of NDV was the greatest twofold 0.5-cc. dilution in physiologic saline causing complete agglutination of 0.5 cc. of a 0.5 per cent suspension of chicken red cells. Red cell suspensions giving HA titers of 1: 640 or 1:1,280 were used for all tests.

Undiluted amnioallantoic fluid (AAF) NDV was used for challenging all chickens in this study. The infective AAF was harvested from several embryos that died thirty-six to forty-eight hours after inoculation, pooled, mixed thoroughly, divided into 5-cc. aliquots, and stored in screw cap tubes at −48 C. A separate tube of virus was used for each experiment. Embryo infectivity titrations of NDV used for challenging chickens were made by allantoic inoculation of 10-day-old chicken embryos with 0.05 cc. of tenfold dilutions of infective AAF. Four embryos were used for each dilution, and the embryo $l.d._{50}$ (lethal dose) was calculated by the method of Reed and Muench.[6] The embryo infectivity titrations were made to determine the quantity of active virus employed in the intramuscular and intranasal inoculations. It is not implied that the embryo $l.d._{50}$ titers have any relation to the chicken pathogenicity of the NDV strains employed in this study. Chickens hatched from susceptible hens were employed for obtaining control data on pathogenicity of the challenge viruses. Since the principal objective of this study was to obtain information on the behavior of NDV in groups of chickens hatched from vaccinated or recovered hens, the actual embryo or chicken $l.d._{50}$ of the virus doses was not of great importance. Also, the fact that 60 per cent of the experimental chickens were exposed by direct contact with the inoculated birds for the purpose of obtaining information on the chicken-to-chicken transmissibility of NDV in parentally immune chickens reduces the significance of $l.d._{50}$ titrations of the challenge viruses. We know of no means by which the number of

From the Department of Animal Pathology, Kentucky Agricultural Experiment Station, University of Kentucky, Lexington.

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either chicken or embryo l.d.$_{50}$ units obtained by chickens under natural or experimental conditions of contact exposure may be ascertained. Obviously, the natural spread of ND is not regulated by known numbers of chicken or embryo l.d.$_{50}$ units.

The SN tests employed 0.05 cc. per embryo of phenolized chicken serum and an equal quantity of appropriate tenfold dilutions of NDV Ky-127 per embryonated egg. The serum-virus mixture was incubated one hour at room temperature before inoculation into the allantoic sac of 4 embryos for each virus dilution. For egg yolk, a 1: 5 dilution in physiologic saline was used for determining embryo infectivity neutralization.

The B1 strain of NDV used for vaccination of the hens supplying chickens for the parental immunity experiments was undiluted AAF harvested from infected embryos dying on the third and fourth days after inoculation. In the vaccination procedure, 0.05 cc. of the undiluted infective AAF was instilled into one nostril of each chicken. The vaccine virus was stored in rubber-stoppered serum bottles for one to three months at −20 C. before use. The wing-web vaccinations were made with two brands of commercial live virus vaccines. The dried virus was reconstituted according to package directions, and one stab was made through the wing web using the double pronged "inoculator" supplied with the vaccine.

For definitive recording of results of the challenge exposures, the chickens that died and also those showing nervous symptoms and paralysis at twenty-one days after exposure were used. Since chickens showing nervous symptoms and paralysis have little chance of developing into useful animals, they were counted as dead in estimating the total mortality. Although 5 to 10 per cent of surviving chickens were severely stunted following exposure with the GB strain of NDV, they were regarded as having resisted the infection.

Excluding the hens used for experiments 1 and 2 (table 1), no attempt was made to determine the SN or HI activity of the serum and egg yolk of the individuals supplying the eggs for this study. Chickens from the vaccinated hens were divided by random selection into groups for different routes of exposure. Attempts were not made to group the chickens according to SN or HI titers of their serum.

RESULTS

The chickens used for experiments 1 and 2 (table 1) were hatched from hens that survived a natural outbreak of ND. Eggs for hatching were obtained between 256 and 305 days after egg production had fallen to zero as a result of the disease. During the week preceding the collection of eggs for hatching, the hens were placed in laying batteries, and serum and eggs were obtained from each hen for SN tests. Also, serum and eggs were obtained for SN tests at the end of the period during which chickens were hatched for challenge exposure. During this period, serum from each of the hens neutralized more than 10^6 embryo l.d.$_{50}$ of NDV strain Ky-127 and a 1 : 5 dilution of yolk from eggs laid by the hens neutralized from $10^{3.6}$ to $10^{5.5}$ embryo l.d.$_{50}$. Chickens hatched from these hens were exposed by intranasal instillation of 10^7 embryo l.d.$_{50}$ of NDV Ky-50 at ages of 1, 2, and 3 weeks. Lethal infection did not occur in any of the chickens challenged intranasally with NDV Ky-50, but nervous symptoms were present in 2.5 per cent of the 1- and 2-week-old chickens. In 22 susceptible 7-week-old chickens used as controls, intranasal instillation of 10^7 embryo l.d.$_{50}$ of NDV Ky-50 killed 13.6 per cent and caused nervous symptoms in 18.1 per cent. In other experiments,[7] intranasal inoculation with this lot of NDV Ky-50 caused death and/or nervous symptoms of 100 per cent of 9-day-old chickens, 90 per cent of 16-day-old chickens, and 70 per cent of 30-day-old chickens. Other chickens hatched from the recovered hens were exposed by intramuscular injection of 10^7 embryo l.d.$_{50}$ of the GB strain of NDV at ages of 1, 2, 3, and 4 weeks. This inoculation caused death and/or nervous symptoms in 22.6 per cent of the 1-week-old chickens, 86.8 per cent of the 2-week-old chickens, and 100.0 per cent of the 3- and 4-week-old chickens.

Subsequent to observing the variation

TABLE 1—Results of Challenge Exposure Experiments with Chickens Hatched from Hens Recovered from Newcastle Disease Virus Infection

Experiment	Days from infection of hens	Method of challenge exposure of chickens	Strains of NDV used for challenge	Dose of virus*	Age of chickens (days)	No. of chickens	Mortality (%)**
1	270	IN	Ky-50	10^7	7	77	2.5
	263	IN	Ky-50	10^7	14	78	2.5
	256	IN	Ky-50	10^7	21	20	0.0
	Controls+	IN	Ky-50	10^7	34	22	31.7
2	305	IM	GB	10^7	7	31	22.6
	298	IM	GB	10^7	14	38	86.8
	291	IM	GB	10^7	21	29	100.0
	286	IM	GB	10^7	28	36	100.0

+Chickens hatched from susceptible hens. IN=intranasally. IM=intramuscularly. *Embryo l.d.$_{50}$. **Percentage of mortality includes chickens showing nervous symptoms and paralysis at twenty-one days after exposure.

with intranasal and intramuscular exposure in the preceding experiments, a parental immunity challenge procedure was developed to provide data on comparative susceptibility to intranasal and intramuscular inoculation. In an attempt to make the challenge tests comparable to natural exposure, other chickens were exposed by direct contact with those inoculated intranasally and intramuscularly. The chickens were challenged in groups of 100 or more; 20 per cent were inoculated intramuscularly, 20 per cent intranasally, and 60 per cent were exposed by direct contact with those having received the inoculations. In all experiments, the chickens were quartered in electrically heated brooders having a deck floor space of approximately 9.3 sq. ft. For the 1-day-old and 1-week-old chickens, there appeared to be no appreciable difference in mortality if a group of 100 were exposed in one deck of the brooder or if they were divided equally between two decks. Fifty chickens were placed in each deck for all trials subsequent to experiment 4. Since the results of intramuscular exposure with the GB strain of NDV in experiment 2 indicated that the maximal protection derived from yolk-transferred antibody was present during the first two weeks after hatching, chickens were challenged at ages of 2 weeks or less in all subsequent trials.

The hens supplying eggs for experiment 3 (table 2) were hatched from a flock that had had natural infection with NDV. A period of four weeks after hatching was allowed for dissipation of passively transferred antibody before intranasal vaccination with the B1 strain of NDV. When the B1-vaccinated stock came into production approximately six months later, eggs representing four days' produce of the flock were hatched for challenge experiments. The 1-day-old chickens were challenged with the GB strain of NDV. There was no protection in these chickens against intramuscular and intranasal inoculation and only slight protection against contact exposure. Of 63 chickens exposed by direct contact, 93.7 per cent died or developed nervous symptoms.

The parent stock of the chickens used in experiment 4 (table 2) was vaccinated intranasally with the B1 strain of NDV at 4 months of age. Chickens were hatched from one day's production of the flock at 103 days after vaccination. When they were 7 days old, these chickens were challenged with the GB strain of NDV. The mortality resulting from this exposure was 91.6, 76.1, and 73.9 per cent, respectively, for the intramuscular, intranasal, and contact routes.

The parent stock supplying chickens for experiment 5 (table 2) were hatched from susceptible hens and were vaccinated when 10 weeks old by intranasal instillation of

TABLE 2—Results of Challenge Exposure Experiments with Chickens Hatched from Hens Vaccinated Intranasally with the B1 Strain of Newcastle Disease Virus

Experiment	Age of parent stock at vaccination	Days from vaccination of hens	Method of challenge exposure of chickens	Strain of NDV used for challenge	Dose of virus*	Age of chickens (days)	No. of chickens	Mortality (%) **
3	4 weeks	180	IM	GB	10^7	1	24	100.0
		180	IN	GB	10^7	1	36	100.0
		180	CE	GB	..	1	63	93.7
4	4 months	103	IM	GB	10^7	7	36	91.6
		103	IN	GB	10^7	7	35	76.1
		103	CE	GB	..	7	115	73.9
5	10 weeks	150 to 190	IM	GB	10^7	1	20	100.0
		150 to 190	IN	GB	10^7	1	20	95.0
		150 to 190	CE	GB	..	1	60	41.6
		150 to 190	IM	GB	10^7	7	20	95.0
		150 to 190	IN	GB	10^7	7	20	75.0
		150 to 190	CE	GB	..	7	60	41.6
		150 to 190	IM	GB	10^7	14	20	95.0
		150 to 190	IN	GB	10^7	14	20	75.0
		150 to 190	CE	GB	..	14	63	53.4
		Controls+	IN	GB	10^7	35	35	100.0
		Controls+	CE	GB	..	25	25	96.0

IN=intranasally; IM=intramuscularly; CE=contact exposure. *Embryo l.d.$_{50}$. +Chickens hatched from susceptible hens. **Percentage of mortality includes chickens showing nervous symptoms and paralysis at twenty-one days after exposure.

the B1 strain of NDV. The chickens used in the challenge experiments were hatched from eggs laid 150 to 190 days after vaccination. The chickens were challenged with the GB strain of NDV at ages of 1, 7, and 14 days. In the 1-day-old chickens, there was no protection against intramuscular inoculation. The mortality in the intranasal group was 95 per cent and in the contact group it was 41.6 per cent. In the 1-week-old chickens, 95 and 75 per cent, respectively, succumbed to intramuscular and intranasal inoculation, and the mortality in the contact group was 41.6 per cent. The same mortality was obtained in the 2-week-old chickens for intramuscular and intranasal inoculation, and the loss from contact exposure was 53.4 per cent.

The parent stock supplying the chickens used in experiment 6 (table 3) was inoculated in the wing web with a commercial live virus vaccine at 4 months of age. Chickens were hatched from eggs laid between 120 and 150 days after vaccination. The chickens were challenged with the GB strain of NDV at ages of 1, 7, and 14 days. The mortality resulting from intramuscular inoculation was fairly uniform for the three age groups, being 70 per cent for the 1-day-old chickens, 75 per cent for the 1-week-old chickens, and 65 per cent for the 2-week-old chickens. The mortality following intranasal inoculation was quite variable, being 40 per cent in the 1-day-old chickens, 65 per cent in the 1-week-old chickens, and 30 per cent in the 2-week-old chickens. Equal mortality, 35.9 per cent, occurred in the 1-day-old and 2-week-old chickens exposed by contact, and the mortality in the 1-week-old chickens was 57.3 per cent.

The chickens used in experiment 7 (table 3) were hatched from hens receiving wing-web vaccination with a commercial live virus vaccine at 6 months of age. Eggs from which the chickens were hatched were laid 268 days after vaccination. When they were 5 days old, the chickens were challenged with the GB strain of NDV. The mortality from intramuscular, intranasal, and contact exposure was 100, 90, and 64.5 per cent, respectively.

The hens providing chickens for experiment 8 (table 3) were inoculated in the wing web with a commercial live virus vaccine at 6 weeks of age. Eggs from which the experimental chickens were hatched were laid from 210 to 224 days after vaccination. All chickens from this flock of hens were challenged when they were 5 days old, and the respective mortality from intramuscular, intranasal, and contact exposure was 40, 65, and 15.6 per cent. In a similar group of chickens, the mortality resulting from $10^{7.4}$ embryo $l.d._{50}$ of NDV strain Ky-157 was 55 and 30 per cent, respectively, for intramuscular and intranasal inoculation, and the mortality from contact exposure was 20.5 per cent. The same dose of NDV Ky-157 caused death of 95 per cent of 20 susceptible 3-week-old chickens when the virus was injected intramuscularly, and 10^5 embryo $l.d._{50}$ intratracheally caused death of 25 per cent of 10-week-old susceptible chickens. Another group of chickens from this flock was exposed to NDV strain Ky-127. This virus has low pathogenicity, $10^{7.3}$ embryo $l.d._{50}$ intramuscularly failing to cause any deaths in a group of 20 susceptible 3-week-old chickens. The mortality resulting from NDV Ky-127 in the chickens from the vaccinated hens was 10 per cent with intramuscular inoculation, zero with intranasal inoculation, and 4.7 per cent from contact exposure. In susceptible chickens receiving the same exposure, the mortality was 65, 70, and 33.7 per cent, respectively, for intramuscular, intranasal, and contact infection.

Blood samples for HI tests were obtained four weeks after exposure from the chickens that survived the contact infection in experiment 6. Each chicken had an HI titer of more than 1 : 160, the highest serum dilution tested. Similar HI titers were obtained for all survivors of the contact group of experiment 7. The contact survivors of experiment 8 had HI titers of more than 1 : 320. Symptoms of respiratory infection were prevalent in chickens subjected to intranasal inoculation and contact exposure. Examination of the surviving chickens for lesions of ND was not made, since they were retained for other studies.

For control chickens on the intranasal inoculations with the GB strain of NDV, a single group of 35 susceptible chickens was used. These birds were inoculated intranasally with 10^7 embryo $l.d._{50}$ of the GB strain when they were 35 days old. This exposure resulted in death of 34 of the chickens and caused the appearance of

nervous symptoms in the 1 remaining chicken, thus eliminating the entire group (table 2). A group of 25 susceptible chickens 25 days old were exposed by contact in separate sections of growing batteries to obtain control data on pathogenicity of the GB strain of NDV by contact exposure. Of these birds, 96 per cent died and 1 recovered from the infection (table 2). The pathogenicity of the GB strain following intramuscular inoculation was adequately demonstrated by the 100 per cent mortality in the 3- and 4-week-old chickens hatched from recovered hens in experiment 2 (table 1). Since susceptible younger chickens readily succumb to NDV infection in greater numbers, it was obviously unnecessary to introduce susceptible controls 2 weeks old or less for comparison with the chickens hatched from vaccinated hens and challenged intramuscularly, intranasally, or by contact exposure with the GB strain of NDV.

DISCUSSION

A summarization of experiments 3, 4, and 5, in which the GB strain of NDV was employed in challenging chickens for evaluating the yolk-transferred passive protection resulting from intranasal vaccination with the B1 strain of NDV, disclosed a progressive decrease in mortality for intramuscular, intranasal, and contact exposure, respectively. In these trials, all chickens were challenged at ages of 2 weeks or less. The number of chickens was 120, 131, and 361, respectively, for the intramuscular, intranasal, and contact groups, and the respective mortality was 95.8, 84.5, and 63.7 per cent. Summarization of experiments 6, 7, and 8, which employed chickens from wing-web vaccinated hens, showed a similar trend of mortality from infection with the GB and Ky-157 strains of NDV. In these trials, the ages of the chickens were 2 weeks or less. The number of chickens was 120, 120, and 377, respectively, for intramuscular, intranasal, and contact exposure, and the respective mortality was 67.5, 53.3, and 38.9 per cent. Chickens from the B1-vaccinated hens were hatched from eggs laid 103 to 190 days after vaccination, and those from the wing-web vaccinated hens were hatched from eggs laid 120 to 258 days after vaccination. The protection derived from the yolk antibody was obviously greater following wing-web vaccination with the live virus commercial vaccines than from intranasal vaccination with the B1 virus.

TABLE 3—Results of Challenge Exposure Experiment with Chickens Hatched from Hens Vaccinated by Wing-Web Puncture with Commercial Live Virus Vaccines

Experiment	Age of parent stock at vaccination	Days from vaccination of hens	Method of challenge exposure of chickens	Strain of NDV used for challenge	Dose of virus*	Age of chickens (days)	No. of chickens	Mortality (%)**
6	4 months	120 to 150	IM	GB	10^7	1	20	70.0
		120 to 150	IN	GB	10^7	1	20	40.0
		120 to 150	CE	GB	..	1	64	35.9
		120 to 150	IM	GB	10^7	7	20	75.0
		120 to 150	IN	GB	10^7	7	20	65.0
		120 to 150	CE	GB	..	7	61	57.3
		120 to 150	IM	GB	10^7	14	20	65.0
		120 to 150	IN	GB	10^7	14	20	30.0
		120 to 150	CE	GB	..	14	60	39.9
7	6 months	258	IM	GB	10^7	5	20	100.0
		258	IN	GB	10^7	5	20	90.0
		258	CE	GB	..	5	65	64.5
8	6 weeks	210	IM	GB	10^7	5	20	40.0
		210	IN	GB	10^7	5	20	65.0
		210	CE	GB	..	5	64	15.6
		224	IM	Ky-157	$10^{7.4}$	5	20	55.0
		224	IN	Ky-157	$10^{7.4}$	5	20	30.0
		224	CE	Ky-157	..	5	63	20.5
		217	IM	Ky-127	$10^{7.3}$	5	20	10.0
		217	IN	Ky-127	$10^{7.3}$	5	18	0.0
		217	CE	Ky-127	..	5	60	4.7
		Controls+	IM	Ky-127	$10^{7.3}$	5	20	65.0
		Controls	IN	Ky-127	$10^{7.3}$	5	20	70.0
		Controls	CE	Ky-127	..	5	62	33.7

IN=intranasally; IM=intramuscularly; CE=contact exposure. *Embryo l.d.$_{50}$. +Chickens hatched from susceptible hens. **Percentage of mortality includes chickens showing nervous symptoms and paralysis at twenty-one days after exposure.

The mortality in experiment 3 (table 2) was sufficient to indicate almost complete absence of passive protection against the GB strain of NDV. Approximately equal mortality was obtained in experiment 4 (table 2) for the intranasally inoculated and contact exposure groups. In experiment 5, equal mortality, 41.6 per cent, was obtained from contact exposure of the 1- and 7-day-old chickens, and there was an increase of 11.8 per cent to 53.4 per cent in mortality from contact exposure in the 14-day-old chickens. Equal mortality, 75 and 95 per cent, respectively, from the intranasal and intramuscular inoculations, occurred in the 1- and 2-week-old chickens. The failure to demonstrate any difference in mortality between the 1- and 2-week-old chickens with intramuscular and intranasal inoculation suggested the probability of chance selection of individuals obtaining less yolk antibody for the 2-week-old contact chickens. Also, it is possible that chickens in the 2-week-old contact group may have acquired infection a few days after the inoculations were made, permitting greater dissipation of the passive antibody.

An explanation of the variation of mortality in the different age groups from the same route of exposure in experiment 6 (table 3) was not apparent. It could not be attributed to the rate of absorption and dissipation of the yolk antibody. In studying the HI response resulting from wing-web vaccination, we have observed that 10 per cent or more of a flock may not show any HI response at two or three weeks after vaccination, and that some chickens lose detectable HI antibody within three to four months after vaccination. It appears possible that chickens hatched from hens that failed to become immune or those undergoing a very mild reaction may have been the cause of the variable mortality obtained in this trial.

In experiment 8 (table 3), considerable variation of mortality occurred with corresponding routes of exposure, the difference being greater with the GB and Ky-157 strains of NDV. Both viruses were sufficiently pathogenic to cause 100 per cent mortality of 5-day-old susceptible chickens, and although we have found the GB strain capable of killing 70 to 90 per cent of susceptible 10-week-old chickens as compared with 25 per cent mortality from NDV strain Ky-157 in chickens of the same age,[7] this difference in pathogenicity was not apparent in the mortality among the 5-day-old chickens from vaccinated hens. In comparison with the susceptible controls (experiment 8, table 3), the chickens from the vaccinated hens had a very good protection against fatal infection with NDV strain Ky-127. Since no immunologic differences have been found in the three strains of NDV used for challenging the chickens, it appeared that the difference in mortality from NDV strain Ky-127 and the two highly pathogenic viruses resulted from a quantitative modification of the infection in relation to the pathogenicity of the virus. In this respect, it appeared that a quantity of passively transferred antibody adequate for protecting the chickens against NDV strain Ky-127 failed to protect against lethal infection with the two highly pathogenic viruses. It should be recognized that natural factors of host resistance may have been responsible for some of the variation in mortality occurring in this series of experiments.

The absence of uniform passive protection against symptomatic infection of the nervous system or fatal infection by NDV was readily demonstrated in chickens hatched from recovered hens and from hens vaccinated with live virus ND vaccines. Infection of such chickens occurred following intramuscular or intranasal inoculation and contact exposure with four strains of NDV, Ky-50, Ky-127, Ky-157, and GB. The study of Brandly *et al.*[1] indicated that yolk-conferred passive immunity was quite effective in preventing fatal infection from intramuscular inoculation during the first two weeks after hatching. Subsequent intravenous challenge inoculation of their surviving chickens resulted in death of 45 to 58 per cent of the chickens that were previously inoculated intramuscularly when 2 weeks old or less. Since their intravenous challenge inoculation caused 100 per cent mortality in susceptible controls, the chickens that survived the second exposure apparently had acquired sufficient infection from the first inoculation to become immunized. Levine and Fabricant[2] reported that intranasal inoculation caused respiratory infection of chickens hatched from recovered hens and hens vaccinated with a live virus ND vaccine. The respiratory infection occurred in all chickens from 1 day

old upward, regardless of passive SN or HI titers of their serum at the time of inoculation. All of these chickens showed a significant rise of HI titers as a result of the infection.

The chickens in our experiment 1 (table 1) which survived intranasal inoculation with NDV strain Ky-50 were challenged by intramuscular inoculation of 10^6 embryo $1.d._{50}$ of the GB strain of NDV forty-eight days after the first exposure to determine if they had become immune. Of the 1-, 2-, and 3-week-old chickens, respectively, 55, 79, and 95 per cent were immune. The survivors of the 1-day-old chickens subjected to contact exposure in experiment 5 (table 2) were inoculated intranasally with 10^6 embryo $1.d._{50}$ of the GB strain of NDV at 51 days of age and were found to be completely refractory to symptomatic or fatal infection. Hemagglutination-inhibition or SN tests were not made prior to the second exposure. The complete protection, however, indicated that adequate infection resulted from the previous contact exposure to establish immunity.

Brandly *et al.*[1] found that SN titers of chickens hatched from immunized hens decreased rapidly after the second week but that the passively transferred SN antibody persisted in some chickens for forty-three days. Levine and Fabricant[2] found that almost no SN or HI titers were present at seventeen days after hatching in chickens from wing-web vaccinated and recovered hens. For chickens hatched from recovered hens, our own studies[3] showed that the yolk-conferred HI antibody had fallen below significant levels within two or three weeks after hatching. All of the chickens surviving contact exposure in our experiments 6, 7, and 8 had HI titers of more than 1 : 160 or 1 : 320 at four weeks after exposure. At this time, the ages of the chickens varied from 4 to 6 weeks, and passively conferred antibody should have been almost completely dissipated. The HI titers indicated that every surviving chicken had acquired infection from contact exposure. These findings are in agreement with those of Levine and Fabricant.[2]

Infection with the B1 strain of NDV in young chickens hatched from actively immunized hens may occur without symptoms of respiratory infection being present.[3] In the same study, we observed that 3-day-old chickens with yolk-conferred passive immunity did not show a detectable HI response following intranasal inoculation with the B1 strain of NDV. These chickens were refractory to subsequent intranasal inoculation of NDV strain Ky-50, and had HI responses following the challenge exposure that were characteristic of previously infected chickens, indicating that neither symptoms of respiratory infection nor the development of significant HI titers necessarily accompany infection by the respiratory route in parentally immune chickens. The severity of symptoms and the percentage of mortality in comparable groups of chickens with yolk-conferred passive immunity appeared to be related to the pathogenicity of the infecting virus strain. In contrast to the asymptomatic infection resulting from the B1 strain of NDV and the slight symptoms of respiratory disease resulting from infection with the mildly pathogenic strain Ky-127, marked respiratory symptoms accompanied intranasal inoculation and contact exposure with strains Ky-157 and GB. In experiments 1 and 2 with chickens from the same source, intramuscular inoculation of the highly pathogenic GB strain caused mortality 20 to 100 per cent greater than intranasal inoculation of the moderately pathogenic strain Ky-50. The more efficient protection against the mildly pathogenic strain Ky-127 as compared to the more pathogenic strains Ky-157 and GB was also evident in experiment 8. Also, the failure to obtain a detectable HI response following intranasal inoculation with the weakly pathogenic B1 strain as compared to the development of significant HI titers from contact exposure to strains Ky-127, Ky-157, and GB suggest that the passive antibody was more effective in limiting the extent of infection by viruses of low pathogenicity. Extreme limitation of the degree of infection was evident in the failure of some passively immune chickens to become actively immune to subsequent challenge exposure.[3] In these chickens, the infection was either prevented entirely or was sufficiently limited to the respiratory mucosa that there was no stimulation of systemic immunizing reaction.

SUMMARY

Yolk-conferred antibody carried over to the progeny of hens vaccinated with live Newcastle disease (ND) vaccines or re-

covered from natural infection did not prevent experimental infection with Newcastle disease virus (NDV) in chickens under 2 weeks of age. Chickens obtaining significant serum-neutralization (SN) and hemagglutination-inhibition (HI) antibody from the egg yolk readily developed symptomatic infection of the nervous and respiratory systems or succumbed to fatal infection. Respiratory infection occurred without producing symptomatic systemic infection or fatal issue. Infection followed intramuscular or intranasal inoculation and also from experimental contact exposure.

The variable mortality resulting from challenge exposure of chickens hatched from hens vaccinated with different live virus vaccines indicated that survival was related to the quantity of antibody obtained from the egg yolk. Hens vaccinated with the live wing-web vaccines had higher HI antibody titers than those vaccinated intranasally with the B1 strain of NDV. Following similar challenge exposure, chickens hatched from wing-web vaccinated hens had a higher survival rate than those hatched from intranasally vaccinated hens. In groups of chickens hatched from a single flock of vaccinated hens from which one might anticipate a comparable passive transfer of antibody through the yolk to the young chickens, the percentage of mortality and severity of symptoms appeared to be related to the pathogenicity of the infecting strain of NDV.

Asymptomatic infection occurred with weakly or mildly pathogenic strains of NDV. The occurrence of infection was indicated by the development of significant HI titers and by resistance to subsequent challenge exposure with lethal doses of NDV.

Employing the B1 strain of NDV, parentally immune chickens may be infected by the respiratory route without the stimulation of a detectable HI antibody response. The occurrence of infection was confirmed by resistance to subsequent challenge exposure.

In chickens hatched from immune hens, the mortality was greater from intramuscular inoculation than from intranasal inoculation. The mortality from contact exposure was lower than that resulting from inoculation.

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A Study of the Morphology of the Turkey Spermatozoa—with Special Reference to the Seasonal Prevalence of Abnormal Types

WALTER J. WAKELY, M.S.A., and I. L. KOSIN, Ph.D.

Pullman, Washington

IN THE PAST, studies on the morphology of the avian spermatozoon have been limited largely either to chickens or doves. Sampson and Warren[8] investigated the relationship of morphology of the fowl spermatozoa to their fertilizing ability. They concluded that the morphologic defects in chicken spermatozoa are not serious causes of infertility. Parker *et al.*[7] showed, however, that the proportion of abnormal spermatozoa in cock's semen is negatively correlated with fertility, a point of view in agreement with the numerous observations on mammalian semen. The use of the electron microscope was employed by Baylor *et al.*[1] in the study of normal fowl spermatozoa, while Miller[4] and Zlotnik[10] described the process of spermatogenesis in the chicken. The presence of morphologically abnormal spermatozoa was detected by Shrigley[9] in the semen of dove hybrids.

The present authors are not aware of any corresponding studies on the morphology of the turkey spermatozoa, in spite of the fact that the problem of low fertility in certain domesticated varieties of the common turkey (*Meleagris gallopavo*) often assumes serious economic proportions. Consequently, an investigation was undertaken at Washington Agricultural Experiment Station, Pullman, in 1948, to study, among other aspects of the problem, the morphology of normal and of abnormal types of turkey spermatozoa. Special attention was paid to seasonal variations in the prevalence of the different spermatozoon types.

MATERIALS AND METHODS

The turkey toms used for the observations included Broad Breasted Bronze, Beltsville Small White, and males resulting from crosses involving these two breeds.

The age of the birds ranged, at the beginning of the investigation, from 46 to 50 weeks. The birds were kept outside with no artificial illumination provided. Semen was collected from March, 1948 to September, 1948, and was obtained by the massage method of Burrows and Quinn.[2]

The method used for sperm film preparation was that suggested by Munro.[5] This involved diluting the semen samples with Ringer's or physiologic saline solution, flooding the slide with the diluted semen, and fixing it in formaldehyde fumes. No mechanical means were used to distribute the semen over the slide.

Two staining techniques were adopted in staining the spermatozoa. The first method involved the use of Ehrlich's hematoxylin with either acid fuchsin or eosin Y as counterstains. In the second method, Heidenhain's iron hematoxylin and eosin were used. The latter procedure gave satisfactory results, but the preparations thus obtained lacked the quality of clear-cut differentiation of the spermatozoon parts which characterized the films obtained with Ehrlich's hematoxylin.

OBSERVATIONS

The study of the prepared sperm slides was divided into two parts: A) To determine the morphology of a normal spermatozoon, and (B) To observe and study deviations from normality.

All observations were made with an oil-immersion lens with magnification x 1,200. An ocular micrometer was used to measure individual spermatozoa.

A) Normal Spermatozoon.

Similar to other avian spermatozoa, a normal turkey spermatozoon is divided into four major parts: (fig. 1 and 15, 2).

1) Acrosome.—The acrosome is the forepart of the sperm and takes the acid stains. It varies in length from 1.4 to 2.1μ.

2) Head.—Posterior to the acrosome is the head, which varies in length from 9.0 to 12.0μ, with the width at the base about 1.5μ. It takes the basic stains. At the distal end is located the proximal centriole.

3) Body or Midpiece.—The body or midpiece is located posterior to the head. Its sides are parallel and the width is the

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At present, Mr. Wakely is at the Dominion Experimental Farm, Ottawa, Ontario, Canada.

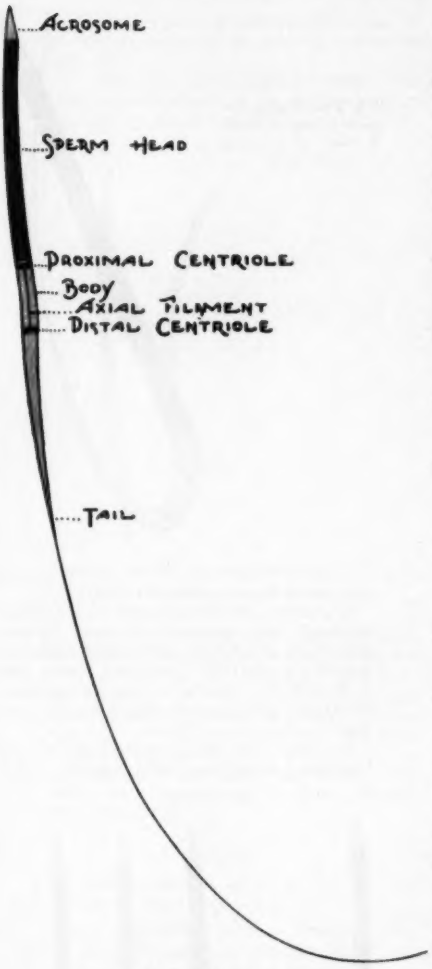

Fig. 1—Normal spermatozoon.

Fig. 2—Variation of normal spermatozoon, showing "rough" condition of body wall.

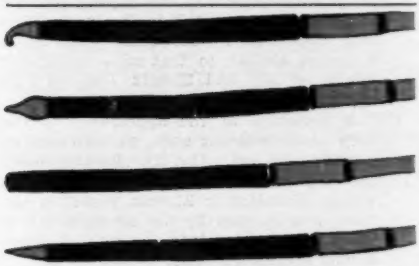

Fig. 3—Variations of acrosome, normal at left.

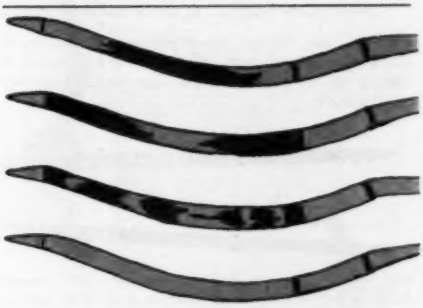

Fig. 4—Variations of "vacuolated" condition of the head.

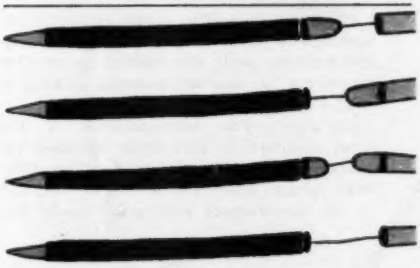

Fig. 5—Variations of filiform condition of the body.

Fig. 6—Giant spermatozoon (top), normal (bottom).

same as the base of the head. The length varies from 3.5 to 5.5μ, and it takes the acid stains. The distal centriole is located at the caudal end. This agrees with Zlotnik's earlier observations[10] on the presence of the two centrioles in the chicken spermatozoon. There was noted in some sperm the presence of a thin line passing through the body from the tail to the head. This was identified as the axial filament, a structure similar to that seen by Miller,[4] Baylor *et al.*,[1] and Zlotnik (*loc. cit.*) in chicken spermatozoa.

A variation in the appearance of the body shape was the same as that seen by Zlotnik (*loc. cit.*) (fig. 2). In both cases, the body was rough in outline, a condition which, according to Zlotnik, is due to certain irregularities in the arrangement of the mitochondria along the axial filament during spermatogenesis.

4) Tail.—The tail is attached to the body at the distal centriole and is generally over 50μ in length. It takes the acid stains.

From the above descriptions and illustrations, it will be seen that the turkey spermatozoon in its gross morphology resembles that of the cock.

Fig. 7—Left: giant-headed spermatozoon with normal body; right: giant-bodied spermatozoon with normal head.

B) Abnormal Spermatozoa.

1) Acrosome.—The most common abnormalities of this structure included the spermatozoa with the hooked or swollen acrosomes or the spermatozoa lacking it (fig. 3).

2) Head.—The abnormalities of the head observed in this study included the presence of "vacuolated" head in the otherwise normal spermatozoa (fig. 4 and 15) or the spermatozoa with giant heads (fig. 7). A few instances of the double-headed spermatozoa were observed (fig. 14).

Fig. 8—Spermatozoon with bent body.

Fig. 9 — Spermatozoon in which body is bent at junction with tail.

3) Body.—The abnormalities of the body included the spermatozoa with filiform-shaped body (fig. 5), bare axial filaments (fig. 5), giant (fig. 7) or bent bodies (fig. 8, 9, and 16), as well as the spermatozoa with the "amorphous"-shaped bodies (fig. 10).

4) Tail.—The abnormalities of the tail included spermatozoa with beaded, coiled,

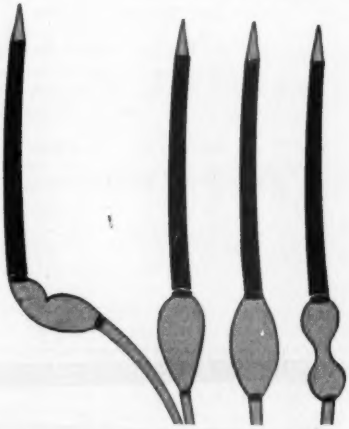

Fig 10—"Amorphous"-shaped bodies.

or abaxial tails. Numerous instances of complete absence of the tail were observed (fig. 11).

5) General Abnormalities.—Under this broad classification can be included abnormalities of the spermatozoon as a whole.

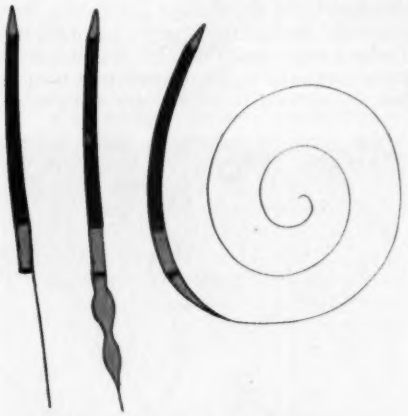

Fig. 11—Variations of tail structure.

These will include the following conditions observed in the present study: immature spermatozoa (fig. 13, 15, and 16) and giant spermatozoa (fig. 6). The spermatozoa showing the "reverse"-staining reaction (fig. 12) can also be listed here. The latter condition is characterized by the head of the spermatozoon taking the acid stains, while the acrosome and the body take the basic stains.

DISCUSSION

The most common abnormality of the sperm head in this study was that referred to as the vacuolated type. Whether this abnormality is similar to the cytolyzed condition of the stored fowl spermatozoa mentioned by Kosin[3] was not established with certainty. One is tempted to surmise that the vacuolated cells observed in this study were undergoing cytolysis of the nuclear material, and that this process takes place while the spermatozoa are in the lower reaches of the vas deferens. In applying the technique of Burrows and Quinn[2] for securing semen samples, it was not always possible to obtain in a single ejaculation all the semen the bird might have stored in the vas deferens. For example, the bird might have defecated while the sample was being obtained. In such a case, no second attempt was made at that time to obtain the balance of the semen, because of the possible contamination of the latter with feces or urates. Certain toms repeatedly produced incomplete ejaculations, apparently because of nervousness. The sperm that remained in the vas deferens, following such incomplete ejaculations, conceiv-

Fig. 12—Reversed stained spermatozoon with increased width.

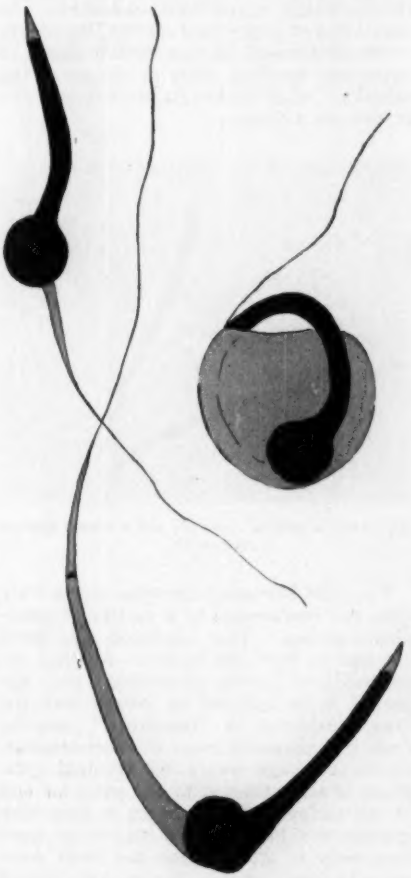

Fig. 13—Immature spermatozoa.

ably could be undergoing cytolysis during the "storage period" before the next ejaculation. Most of these vacuolated-type

Fig. 14—Double-headed spermatozoon.

spermatozoa were obtained during the early part of the test when the birds were being accustomed to artificial ejaculation and when incomplete ejaculations were most frequent. A similar condition leading to cytolysis might also exist in toms that are not aggressive in the breeding pens, with the resultant psychologic castration. In such cases of suppressed mating, the sperm would presumably undergo initial stages of aging and cytolysis while in storage at the relatively high temperature that prevails in the vas deferens.

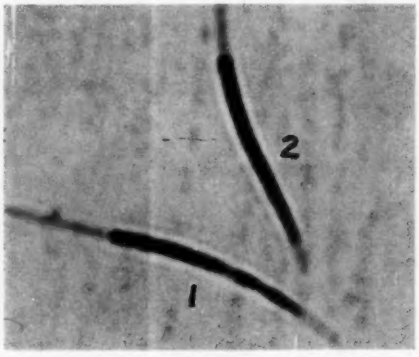

Fig. 15—"Vacuolated" type (1) and a normal spermatozoon (2).

The most prevalent abnormal sperm body type was represented by a variety of amorphous shapes. This condition was most common in July and August. Another abnormality of sperm morphology that appeared to be affected by season was the type considered as "immature," ranging from the spermatid stage of spermatogenesis to the stage where the residual cytoplasm is still attached to the posterior end of the nucleus. The presence of immature spermatozoa in ejaculates was noted most frequently in April, when the birds were sexually most active. During this period of high sexual activity, there is a heavy drain on the reproductive system of breeding toms. It is likely that under such conditions the spermatids are not retained long enough in the semeniferous tubules of the testes to complete the process of spermateliosis. Munro[6] has shown that fowl sperm complete their physiologic maturation during their passage through the vas deferens. Under normal conditions, the sperm mature morphologically in the semeniferous tubules, but, in contrast to physiologic maturation,

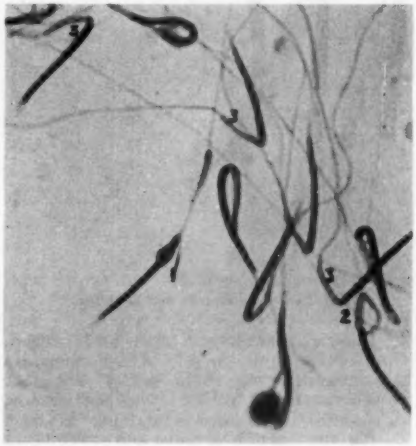

Fig. 16—Immature spermatozoon (1), spermatozoon showing body bent at tail (2), and spermatozoa with bodies bent at head (3).

it would seem that morphologic maturation can not be completed in the vas deferens of the tom.

Two abnormalities that aided in demonstrating the existence of the axial filament in the normal turkey spermatozoa were the filiform condition of the body and the type of body that appeared to have no mitochondrial sheath. In normal turkey sperm, the axial filament was seen to pass through the body from the tail to the head. In the filiform type of abnormal sperm, the sides appeared to be pinched in, leaving a thin line. In the case where the mitochondrial sheath was missing entirely, a thin line was noted to join the tail with the head.

The normal staining reaction of the avian spermatozoon to the Heidenhain hematoxylin and counterstain method of staining

is for the head to take basic stains and the acrosome and the body to take acid stains. In this study, it was noted that during the month of August this staining reaction was frequently reversed. At other times, this condition of reversed staining could be produced by centrifuging the semen and then staining this sperm with the usual sequence of basic and acid stains.

SUMMARY

1) The normal turkey spermatozoon morphologically is similar to other avian types studied and is divided into four parts: acrosome, head, body (midpiece), and tail.

2) Evidence has been presented for the existence of an axial filament in the body of the spermatozoon.

3) A number of abnormal types of turkey spermatozoa have been identified in this study. These include: of the acrosome —hooked, swollen, or absent; of the head— "vacuolated," double headed, giant; of the body—filiform, bent, "amorphous," bare axial filament; and of the tail — beaded, coiled, abaxial, or absent.

4) There appears to be a seasonal trend in the prevalence of the different types of abnormal spermatozoa identified in this study.

5) Ehrlich's hematoxylin is a more satisfactory nuclear stain for the turkey spermatozoa than Heidenhain's iron hematoxylin.

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Characteristics of the B1 Strain of Newcastle Disease Virus

S. B. HITCHNER, B.S., V.M.D.; G. REISING, B.S., M.S.;
H. VAN ROEKEL, D.V.M., Ph.D.

Amherst, Massachusetts

ALTHOUGH THE general characteristics of Newcastle disease virus (NDV) (avian pneumoencephalitis) have been widely studied and reported in the past, the apparent singular nature of the B1 strain of virus seems worthy of recording. This strain of virus was originally received as a strain of infectious bronchitis virus (IBV) from Dr. F. R. Beaudette of the New Jersey Agricultural Experiment Station, and its history has been reported in a previous publication.[4]

PROCEDURE

Virulence in Passively Immune and Nonimmune Embryos.—The low virulence of the B1 strain for baby chicks has been established and recorded.[4] Not only is its low virulence disclosed in the ability of baby chicks to survive its presence but also in its reduced virulence for chicken embryos. Whereas most Newcastle disease (ND) strains of virus kill 10-day-old embryonating eggs in two to three days postinoculation, the majority of embryos inoculated into the allantoic sac with the B1 strain die between the third and fifth days. This mortality pattern is true only when embryos originate from ND-nonimmune stock. The mortality pattern is altered in embryos possessing passive immunity to ND, the mortality being distributed throughout eleven days postinoculation, with the peak mortality around the sixth and seventh days.

Table 1 gives a representative picture of the embryo mortality in two groups of 50 embryonating eggs obtained from ND-immune and -nonimmune flocks.

These eggs were inoculated with 0.1 ml. of a 1:100 broth dilution of allantoic fluid infected with the B1 virus. The 50 controls were inoculated with 0.1 ml. of nutrient broth. The immune eggs were obtained from the Massachusetts Experiment Station flock which had been immunized with a Massachusetts strain of virus[7] by the wing-web method approximately ten months previously. Table 2 shows that similar delay in mortality occurred in eggs which originated from a flock immunized with the B1 strain of virus three and one-half months previously.

The incidence of death in the embryos on the various days postinoculation may vary considerably depending upon the immune status of the parent stock, but the fact that passive antibodies do delay the death of embryos inoculated with the B1 strain seems well established. Alteration of the mortality pattern by passive antibodies with other NDV strains is not known to have been recorded, with the exception of the work reported by Keogh,[5] in which he was able to prolong the life of embryos by using a minimal dose of NDV followed after an interval by immune serum. What Keogh performed experimentally is demonstrated naturally with passively immune eggs and the B1 strain.

TABLE 1—Mortality Following Inoculation of 10-Day-Old Newcastle Disease-Immune and -Nonimmune Embryos with B1 Strain of Newcastle Disease Virus

ND status of laying flock	No. eggs inoc.	No. deaths Days after inoculation										
		1	2	3	4	5	6	7	8	9	10	11
ND-immune	50	2	0	4	4	4	12	6	7	7	2	2
ND-nonimmune	50	0	1	6	29	13	0	1	..	..	..	..
Controls ND-immune	50	1	0	0	0	1	0	0	0	0	0	0

Comparative Rate of Propagation.—In order to determine whether the presence of ND antibodies in the embryos interfered appreciably with virus propagation, the comparative growth rates of the B1 and California 11914 strains of virus were studied in embryos from ND-immune and -nonimmune flocks. Ten-day-old embryos were used throughout this study. Sixty eggs from ND-immune and -nonimmune flocks, each, were inoculated with $10^{3.75}$ embryo lethal doses (e.l.d.'s) of strain California 11914. One hundred eggs from the immune and nonimmune flocks, each, were inoculated with $10^{3.39}$ e.l.d.'s of the B1 strain. Following inoculation, all eggs were candled at one, six, and twelve hours and, thereafter, at six-hour intervals for a period of four days.* At each candling period, 5 live embryos from each inoculated group were removed and placed in the refrigerator. After storage in the refrigerator for six hours, the fluids were harvested and placed in a freezer. When egg sterility tests were completed, 1-ml. samples from each

Contribution No. 778 from the Massachusetts Agricultural Experiment Station, Amherst.

*Last candling was made at ninety-four instead of at ninety-six hours.

of the five tubes of each group were pooled. These pooled samples were maintained in a frozen state at −25 C. until titrated later in eggs from a ND-nonimmune flock. Four eggs were used for each dilution and the 50 per cent end point computed according to the method of Reed and Muench.[8] All the pools were not titrated, but the titers of a sufficient number were determined to permit plotting a growth curve (chart 1).

The results reveal that there is very little difference in the growth rate of the B1 and California 11914 strains when propagated in ND-immune and -nonimmune embryos. There was a slight lag of approximately two hours in the B1-inoculated ND-immune eggs, but the passive immunity present had no significant effect upon the final strain titer. All embryos inoculated with the California strain of virus, in both the immune and the nonimmune groups, were dead at the end of forty-eight hours. The titration on the 54-hour harvest, shown for each of these groups, was made on a five-egg pool from eggs which were left in the incubator for six hours after death. The virus titers were $10^{9.0}$ and $10^{8.5}$ for the immune and nonimmune embryos, respectively. At the end of the fourth day, only 2 embryos remained alive in the B1-nonimmune group, whereas, 21 remained alive in the immune group. Five embryos of the nonimmune group which were dead at the ninety-fourth hour were allowed to remain in the incubator eight hours before being harvested and pooled. This pool gave a virus titer of $10^{9.7}$, verifying the ability of NDV to withstand incubator temperatures for considerable periods without loss of infectivity. Five of the B1-inoculated embryos in the immune group were still alive at the end of the sixth day postinoculation. The fluids from these eggs were harvested, pooled, and titrated as in the other harvests. The titer of this pool was $10^{9.3}$.

TABLE 2—Mortality Following Inoculation of 10-Day-Old Newcastle Disease-Immune and -Nonimmune Strain of Newcastle Disease Virus

ND status of laying flock	No. eggs inoc.	No. deaths Days after inoculation 1	2	3	4	5	6	Survived
Nonimmune	18	2	1	7	7	1	0	0
Immune to B1 strain	18	0	0	0	2	1	7	8—curled and stunted
Immune to U. of Massachusetts strain	18	0	4	1	1	1	5	6—curled and stunted

Hemagglutination.—The B1 strain of virus gives good agglutination of chicken erythrocytes, frequently agglutinating cells in a dilu-

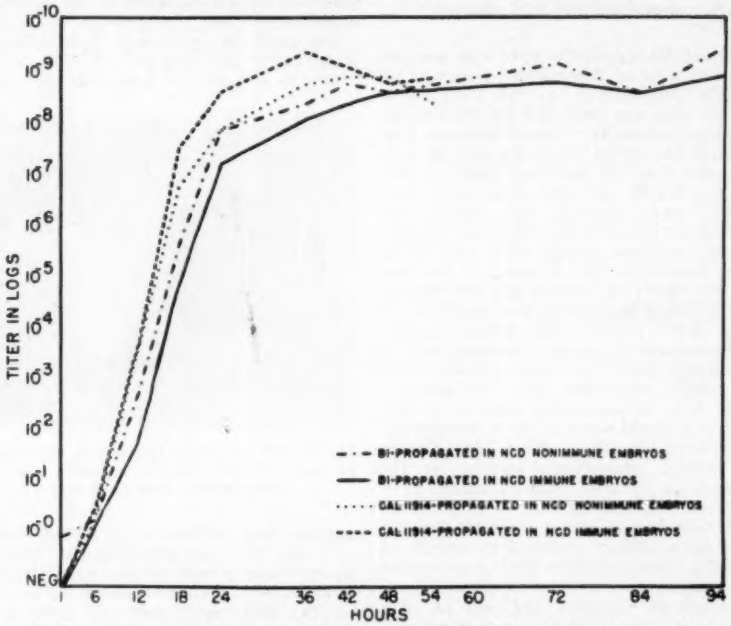

Chart 1—Comparative rates of propagation of the B1 and California 11914 strains of Newcastle disease virus in Newcastle disease-immune and -nonimmune embryos.

tion of 1:2,560. This strain is used routinely for running hemagglutination-inhibition (HI) tests in the laboratory. Because of its low virulence, it can be used with relative safety in the laboratory without formalinization. Embryonating eggs inoculated with the B1 virus give high yields of allantoic fluid, averaging 5 to 10 ml. per egg, which facilitates the collection of fluid for the HI test. As much as 15 ml. of fluid has been harvested from an individual egg.

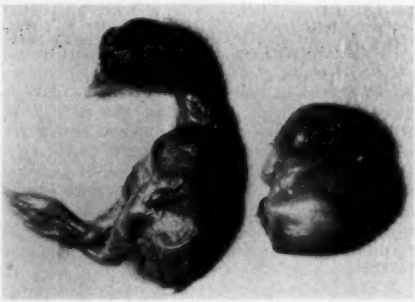

Fig. 1—Right: 15-day-old live embryo inoculated when 10 days old with B1 strain of Newcastle disease virus. Left: noninoculated control, 15 days old.

Lesions of Embryos.—Embryos that die two to three days after inoculation with the B1 virus show reddening of the feet and congestion of the yolk sac, liver, and kidney, but the hemorrhages which are frequently seen with some other strains of NDV are seldom seen with the B1 strain at this time. Embryos that die on the fourth and succeeding days postinoculation show stunting and curling, not unlike that attributed to IBV (fig. 1 and 2). The curling and stunting become more pronounced with the lapse after inoculation. In the late stages, the shriveled embryo has the appearance of a tissue dehydrated and hardened in formalin. When death occurs during this period, hemorrhages are found frequently on the chorioallantoic sac or yolk sac. The amnionic sac is tightly enveloped about the embryo; amnionic fluid is practically nonexistent and the yolk is concentrated to a thick consistency. These factors, plus the apparent dehydration of the embryo, undoubtedly account for the high yields of allantoic fluid. It seems probable that curling and stunting of the embryos infected with the B1 strain, as well as with IBV, are not a primary result of the growth of virus in the tissue, but more likely a secondary change brought about by the flow of fluids from the amnionic sac, embryo, and yolk sac into the allantoic cavity. Cunningham and El Dardiry,[2] in their studies on the distribution of IBV in embryonating eggs, found the greatest concentration in the chorioallantoic membrane, followed in order by the allantoic fluid, amnionic fluid, and liver. It is clearly evident that the normal physiologic development of the embryo has been altered, and it is possible that the higher concentration of virus in the allantoic sac may increase the osmotic pressure of the allantoic fluid inducing a flow of fluids into this sac. The curling and stunting, therefore, may be caused secondarily by the dehydration process. Further investigation is necessary for proof of this explanation.

Purity of B1 Strain.—The possibility of the B1 strain being comprised of a mixture of IBV and NDV has been questioned on several occasions. If IBV does coexist with NDV, there is no evidence to date that it has ever exerted itself in the form of a disease syndrome in chicks. To demonstrate the coexistence of the viruses meets with certain difficulties in view of the apparent loss of pathogenicity and antigenicity for chicks of the Beaudette embryo-killing strain of IBV.[1] Since there is no means of demonstrating its presence on the basis of pathogenicity or antigenicity in chicks, the only plausible approach to separating the two would be to neutralize the NDV with immune serum. If this could be accomplished successfully, and embryos continued to die in the absence of hemagglutination of the allantoic-amnionic fluid, then the presence of the two viruses could be confirmed. Four attempts to demor rate the presence of IBV in the B1 strain by this method have been made without success. As a control on the method employed, IBV and NDV were intentionally mixed and passed through eggs for four generations. Four attempts were made to separate the two viruses by the previously mentioned method, using fluid from the first, second, third, and fourth passages, but these also were without avail.

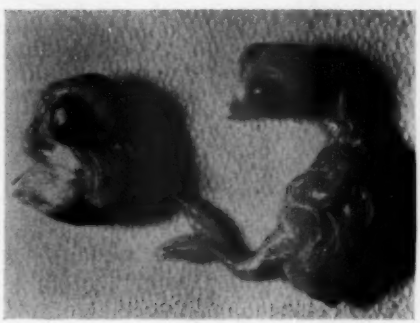

Fig. 2—Left: 15-day-old live embryo inoculated when 10 days old with infectious bronchitis virus. Right: noninoculated control, 15 days old.

Further trials were not made because of the exhaustion of the supply of immune serum. A more complete study of the technique employed seems warranted before any conclusions are drawn from this method of approach. One clue to the improbability that the behavior of the B1 strain is due to a mixture of viruses is set forth by the fact that fluid harvested from embryos dying in the 10^{-9} dilution will produce the curling and stunting characteristic of the B1 strain when transferred back into embryos. This dilution exceeds the embryo infective titer of known IBV strains that have been reported.

DISCUSSION

The curling and stunting caused by the B1 strain of NDV negate the observation made by Fabricant[3] that curling is a characteristic change, pathognomonic of infectious bronchitis (IB). Due to the widespread use of the B1 strain in the intranasal vaccine and the possibility of recovering it upon attempts at virus isolation, the diagnosis of IB can not be made on the basis of the curling and stunting of the embryos, alone, but must be substantiated by the absence of hemagglutination by the allantoic fluid.

Another fact brought to light by study of the B1 strain is the importance of knowing the immune status of the laying flock supplying eggs for laboratory tests. Passive immunity may have more effect on virus isolation results in such diseases as ND and IB than has heretofore been surmised, and the availability of eggs from susceptible flocks is becoming increasingly important in laboratory studies. If eggs from ND-immune flocks must be used for titration studies with the B1 strain of virus, mortality alone can not be a criterion of infectivity unless the observation period is prolonged through the twenty-first day. If it is more desirable to terminate the observation period at the end of six days, as is ordinarily done, then the remaining live embryos must be opened to observe characteristic pathologic changes, and the allantoic fluid tested for hemagglutination, to determine the end point of virus infectivity.

SUMMARY

The low virulence of the B1 strain of Newcastle disease virus (NDV) was disclosed not only in the ability of baby chicks to withstand its presence but also in its reduced virulence for embryos. Embryos from NDV-nonimmune flocks usually died three to five days postinoculation. When embryos possessed passive antibodies, the mortality might be delayed eleven days postinoculation. Although mortality was delayed, there was no significant reduction in the virus titer of the allantoic fluid.

The B1 strain gave good agglutination of erythrocytes in dilutions as high as 1 : 2,560.

Embryos exhibited lesions similar in many respects to those caused by infectious bronchitis virus (IBV). The curling and stunting of the embryos were the changes observed most consistently. Attempts to verify the coexistence of IBV with the NDV have been negative.

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Studies of Newcastle Disease and Poliomyelitis Viruses in Rhesus Monkeys

REGINALD L. REAGAN; MARY G. LILLIE, M.S.; DOROTHY M. SCHENCK, M.S.; L. J. POELMA, D.V.M.; A. L. BRUECKNER, V.M.D.

College Park, Maryland

IN A RECENT publication, Reagan *et al.*[1] reported that, following the adaptation of the virus of Newcastle disease, California strain No. 11,914, to the Syrian hamster,[2] the modified virus produced symptoms involving the central nervous system in a Rhesus monkey. These symptoms were of the type of central nervous system involvement caused by poliomyelitis virus (PV) infections in the same species. In another paper,[3] it was reported that, in monkeys injected with Newcastle disease virus (NDV), there was evidence of slight resistance to active PV, Brunhilde strain. The results of these preliminary studies prompted the following experiments.

MATERIALS AND METHODS

In one experiment, 32 Rhesus monkeys, weighing approximately 3 lb. each, were exposed by intracerebral injection of 1 cc. of 1 : 1,000 dilution of PV suspension, Brunhilde strain. The poliomyelitis challenge virus, Brunhilde II, supplied by Dr. Howard A. Howe of the Poliomyelitis Research Center, Johns Hopkins University, had a 50 per cent end point of $10^{-6.8}$ in Rhesus monkeys by intracerebral injection. Twenty-eight of the 32 poliomyelitis-inoculated monkeys showed typical poliomyelitis symptoms of central nervous system involvement, and 18 of these died of the disease. The other 4 inoculated monkeys failed to exhibit symptoms of central nervous system involvement.

The surviving 10 monkeys which had shown symptoms of poliomyelitis infection and the 4 monkeys which failed to exhibit symptoms were bled from the heart twenty-seven days after the injection of the PV. The serums were used in chicken embryo-neutralization studies with NDV. No NDV-neutralizing antibodies were found in any of the 14 serums tested. On the twenty-seventh day after the poliomyelitis inoculation, the 14 surviving monkeys and 2 normal controls were challenged intracerebrally with 0.5 cc. of a 10 per cent brain suspension of the 200th passage of hamster-modified NDV. This titrated $10^{-4.8}$ in 3-week-old hamsters by intracerebral injection.[4] The PV had been injected to the right of the midline of the brain of the test monkeys, while the hamster-adapted NDV was inoculated to the left of the midline.

The results obtained in the first experiment after the poliomyelitis-exposed monkeys were challenged with the hamster-adapted NDV are given in table 1. Five of 10 monkeys which had shown symptoms of central nervous system involvement from PV injection became moribund and died between the seventh and nineteenth days following injection of the hamster-adapted NDV, and the remaining 5 survived the challenge virus with no apparent symptoms of central nervous system involvement. This indicated that 50 per cent of those monkeys which recovered from poliomyelitis infection had sufficient acquired or natural resistance or that there was blocking action to cause them to withstand the hamster-adapted NDV challenge. All 4 of the monkeys which had shown no symptoms of central nervous system involvement following poliomyelitis injection succumbed after the hamster-adapted NDV inoculation. Sabin and Olitsky[5] and Harford and Bronfenbrenner[6] found that antibodies developed irregularly in monkeys on recovery from poliomyelitis infection and were not detected until a lapse of two to three months. The interval between poliomyelitis and hamster-adapted NDV challenges may have been too short in the above experiment. Both normal monkeys not previously exposed to PV died after revealing typical symptoms of central nervous system involvement caused by the intracerebral injection of hamster-adapted NDV.

In a second experiment, a total of 14 Rhesus monkeys, weighing approximately 5 lb. each, were used. Four of the monkeys (No. 1, 2, 3, and 4) were immunized with the Lansing strain of PV by giving varying amounts of a 1 : 250 dilution of the virus intramuscularly in the hind legs according to the schedule given in table 2. Because monkey 1 had nervous symptoms involving the central system one month after the initial vaccination, it was discarded. The remaining 3 monkeys which showed no symptoms of central nervous system involvement were challenged intracerebrally one week after the last intramuscular injection

From the Maryland State Board of Agriculture, Live Stock Sanitary Service, University of Maryland, College Park.

TABLE 1—Effect of a Modified Newcastle Disease Virus on Poliomyelitis-Exposed Rhesus Monkeys

Monkey (No.)	Observations on days following NDV challenge																										
	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
63	N	N	N	S	D	M	D																					
47*	N	S	S	M	M	M	D																					
18**	N	S	S	S	Z	Z	D																					
86	N	Z	Z	Z	Z	Z	Z	Z																				
55	N	Z	Z	Z	Z	Z	Z	Z																				
40*	N	Z	Z	Z	Z	Z	Z	Z																				
39*	N	Z	Z	Z	Z	Z	Z	Z																				
37	N	Z	Z	Z	Z	Z	Z	Z																				
10	N	Z	Z	Z	Z	Z	Z	Z																				
23	N	Z	Z	Z	Z	Z	Z	Z																				
25	N	Z	Z	Z	Z	Z	Z	Z																				
67	N	Z	Z	Z	Z	Z	Z	Z																				
81**	N	Z	M	Z	Z	Z	Z	Z																				
68**	N	Z	N	D	N	N	N	N	N	N	S	M	D	D	M	M	D	M	D									

N = appeared normal; S = symptoms involving the central nervous system; M = moribund; D = dead; * = no poliomyelitis symptoms following intracerebral injection with poliomyelitis virus; ** = controls.

with 1 cc. of a 1 : 250 dilution of the Lansing strain of PV. This virus titered 10^{-3} in mice by intracerebral injection. Monkey 2 had typical symptoms involving the central nervous system twelve days after intracerebral inoculation.

TABLE 2—Summary of Poliomyelitis Virus Inoculations Prior to Challenge

Day of vaccination	PV (Lansing strain) vaccine	
	Amount (cc.)	Dilution
1	0.5	1 : 250
3	1.0	1 : 250
7	1.5	1 : 250
10	1.5	1 : 250
17	1.5	1 : 250
24	1.5	1 : 250
31	1.0	1 : 250
35	1.0	1 : 250

On the twenty-eighth day after the poliomyelitis challenge, monkeys 3 and 4 were then challenged intracerebrally with 2 cc. of a 10^{-1} dilution of hamster-adapted NDV, 272nd subculture. Both monkeys showed symptoms of central nervous system involvement and died the seventh and eighth days after the symptoms appeared.

TABLE 3—Summary of Newcastle Disease Virus Inoculations Prior to Challenge

Chicken embryo-adapted NDV vaccine	
Amount (cc.)	Dilution
1.0	1 : 5
2.0	1 : 5
1.5	2 : 5
1.5	2 : 5
1.0	3 : 5
1.0	3 : 5
1.0	3 : 5
1.0	3 : 5

Rhesus monkeys 5, 6, 7, and 8 were immunized by intramuscular injection of chicken embryo-adapted NDV of the nineteenth subculture, as shown in table 3. One week after the last intramuscular injection, the Newcastle-immunized monkeys were inoculated intracerebrally with 1 cc. of a 10^{-1} dilution of the 250th subculture of hamster-adapted NDV which titrated 10^{-4} in young hamsters by intracerebral inoculation. All 4 of these monkeys remained normal in appearance during a thirty-day observation period. At the end of this time, the monkeys were challenged with 0.5 cc. of a 1 : 500 dilution of the Lansing strain of PV. Within ten days following challenge, the 4 monkeys had symptoms of central nervous system involvement.

The remaining 6 normal monkeys were used as controls. Three of the monkeys were challenged intracerebrally with 0.5 cc. of a 1 : 500 dilution of the Lansing strain of PV and 3 were challenged intracerebrally with 2 cc. of a

10^{-1} suspension of hamster-adapted NDV of the 272nd subculture. Observation of the three groups of monkeys are given in table 4.

TABLE 4—Response of Monkeys in Experiment 2 to Challenge Virus

Monkey (No.)	Immunization (type)	Challenge virus (type)	Observations: Days following challenge 2	3	4	5	6	7	8	9	10	11	12
3	Poliomyelitis virus, Lansing strain	Hamster-adapted Newcastle disease virus	N	N	N	S	S	S M	D	..	..	..	..
4			N	N	N	S	S	D	....		..	..	..
5	Chicken embryo-adapted Newcastle disease virus	Poliomyelitis virus, Lansing strain	N	N	N	N	S	S	S D	..	..	..	..
6			N	N	N	N	N	N	N	S	S	S D	..
7			N	N	N	N	N	N	N S	N	S	S	S D
8			N	N	N	N	S	S	S D	..	..	..	..
9	Normal, nonimmunized	Hamster-adapted Newcastle disease virus	N	N	N	N	N	S	S	M D	..	..	..
10	Normal, nonimmunized		N	N	N	S	S	S	S	S	S	D	..
11	Normal, nonimmunized		N	N	N	S	S	S	M D	..	..	..	..
12	Normal, nonimmunized	Poliomyelitis virus, Lansing strain	N	N	N	N	N	S	S	D	..	..	..
13	Normal, nonimmunized		N	N	N	N	N	N	S	S	D	..	..
14	Normal, nonimmunized		D—atypical death										

N = normal; S = symptoms involving the central nervous system; M = moribund; D = dead.

DISCUSSION

In experimentally injected Rhesus monkeys, the modified NDV produced nervous symptoms similar to those of experimentally injected chickens in which the egg-adapted virus, California strain No. 11,914, was used subcutaneously. The monkeys showed irritability and malaise, followed by partial paralysis of the extremities or partial paralysis of one side. The amount of paralysis varied with the individual monkey and, after paralysis was evident, the monkey became moribund within twelve to twenty-four hours. During the moribund period, there was a convulsive type of nervous jerk of the head, or of one limb or both limbs of one side. These convulsive movements usually occurred rhythmically with labored breathing.

SUMMARY

The nervous symptoms caused by intracerebral challenge of hamster-adapted Newcastle disease virus (NDV) in Rhesus monkeys are similar to those observed in experimentally infected chickens inoculated subcutaneously with egg-adapted NDV, California strain No. 11,914.

In the first experiment, all control monkeys and 50 per cent of those monkeys which had recovered from infection with the Brunhilde strain of poliomyelitis virus (PV) succumbed after intracerebral inoculation with hamster-adapted NDV. Fifty per cent of those monkeys which recovered from poliomyelitis infection had sufficient acquired or natural resistance or there was blocking action to cause them to withstand the hamster-adapted virus challenge.

In the second experiment, 2 Rhesus monkeys definitely immune to the Lansing strain of PV failed to withstand intracerebral challenge with hamster-adapted NDV, and 4 Rhesus monkeys immune to NDV failed to withstand intracerebral challenge of the Lansing strain of PV.

From the above experiments, it appears that the hamster-adapted NDV produced Newcastle disease in monkeys inoculated intracerebrally. There was evidence of slight resistance against NDV in those monkeys which had been injected with PV, Brunhilde strain, but not in those vaccinated with the Lansing strain of PV.

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Trace Element Supplements for Asparagine Brands Found to be Deficient for the Growth of Tubercle Bacilli

L. A. BAISDEN, M.S., Ph.D.

Washington, D. C.

ASPARAGINE has been used since 1929 as a satisfactory source of nitrogen in the synthetic medium originally designed by Henley[1] for use in growing the tubercle bacilli from which is derived the tuberculin used by the Bureau of Animal Industry (BAI) in its program to eradicate tuberculosis from all herds of cattle in this country. On this medium, and on a modification of it in which the percentage of asparagine is increased from 1.2 to 1.4 per cent, the amount of growth of the tubercle bacilli has regularly been about 2.0 Gm. dry weight per 100 ml. of medium. The culture usually floats on the surface of the medium for seven to nine weeks. However, within the past year, three commercial brands of asparagine were purchased which failed to afford satisfactory growth when used as a source of nitrogen in the BAI medium. Cultures on batches of mediums made of the deficient brands usually grew only enough to cover the surface and then became water-logged and sank after two to three weeks.

Henley[2] found that trace quantities of zinc were required for satisfactory growth on ammonium malate mediums made from certain batches of malic acid. It seemed possible that the failure of recently purchased brands of asparagine to produce good growth was due to the lack of traces of unknown elements. An investigation of this possibility was therefore undertaken.

PROCEDURE

The BAI medium is regularly prepared with distilled water. Since tap water contains traces of many elements, a number of tests of batches of medium prepared with tap water were first made. Two of the deficient brands of asparagine were used in these tests. The available amount of the third was too small for use. On some batches, increased growth was obtained, indicating that needed elements may have been supplied in these cases. However, results were not consistent, possibly because the mineral content of the tap water drawn on different days varied.

More exact information was, therefore, necessary regarding the identity of the mineral supplements that might be needed. The procedure adopted to obtain this information was to analyze the ash of the bacilli, a procedure first used by de Schweinitz and Dorset[3] for determining the gross mineral requirements of

TABLE I—Mineral Supplements to the BAI Synthetic Medium* Prepared with the Use of Certain Brands of Commercial Asparagine Found Unable To Afford Normal Growth of Tubercle Bacilli Used in Tuberculin Production

Aluminum chloride** ($AlCl_3$), pure	0.10 Gm.***
Barium chloride ($BaCl_2 \cdot 2H_2O$), C.P.	0.10 Gm.
Boron as sodium borate ($Na_2B_4O_7 \cdot 10H_2O$), U.S.P.	2.00 Gm.
Copper sulfate ($CuSO_4$) (anhydrous), C.P.	0.20 Gm.
Calcium chloride ($CaCl_2$), (anhydrous), C.P.	5.00 Gm.
Lead acetate, basic ($3Pb(C_2H_3O_2)_2 2PbO$), C.P.	0.10 Gm.
Manganese chloride ($MnCl_2 \cdot 4H_2O$), C.P.	1.00 Gm.
Molybdic acid anhydride (MoO_3), C.P.	0.20 Gm.
Nickel chloride ($NiCl_2$, crystal), C.P.	0.10 Gm.
Silver nitrate ($AgNO_3$), C.P.	0.10 Gm.
Sodium chloride (NaCl), C.P.	10.00 Gm.
Strontium chloride ($SrCl_2 \cdot 6H_2O$), C.P.	0.10 Gm.
Zinc sulfate ($ZnSO_4 \cdot 7H_2O$), C.P.	2.00 Gm.
Cobalt nitrate ($CoNO_3 \cdot 6H_2O$), C.P.	0.10 Gm.

*The BAI medium is modified in this work to contain 1.4 per cent asparagine and 0.196 per cent sodium citrate. The latter constituent is increased over the amount ordinarily used in order to prevent a bothersome precipitate resulting from the addition of the minerals.

**The solid compounds are added to the constituted medium and the mixture stirred well to insure their solution. The finished medium is sterilized in 100-ml. amounts contained in 300-ml. Erlenmeyer flasks for a period of 25 minutes at a temperature of from 115-117 C.

***The amounts given are added to a 72-liter batch of medium.

From the Pathological Division, Bureau of Animal Industry, Agricultural Research Administration, U. S. Department of Agriculture, Washington, D. C.

the organism. Since analyses by chemical means, even with the use of micromethods, seemed unlikely to detect all of the trace elements that may be present in a normally developed culture of tubercle bacilli, a spectroscopic analysis of the ash was made.*

The ash for this purpose was obtained from tubercle bacilli grown on a batch of medium made of a brand of asparagine that afforded adequate growth. The cultures were sterilized by steaming and the growth filtered and washed thoroughly. The organisms were ashed at temperatures less than 500 C. in preparation for the spectroscopic analysis.

Those mineral elements found to be present in the ash, and not supplied in large quantities by the medium, are shown in table 1 and were used as supplements in batches of medium made of various brands of asparagine. The amounts used are also shown in table 1. Cobalt was added, also, since it is present in most vitamin B_{12} preparations,[4] and B_{12} has been found useful in the nutrition of certain bacteria. The compounds furnishing the elements were added either as solutions or as solids to the finished batches before the medium was dispensed in the culture flasks.

Small-scale experiments using both brands of the deficient asparagine were first carried out. The cultures used were the PN, C, and DT strains of the human type of tubercle bacilli. These are the strains regularly used to produce BAI tuberculin. Good growth was consistently obtained, as shown in table 2 which also includes some of the results obtained when tap water was used to supply trace elements.

Following these findings, ten 72-liter batches of medium prepared from each of the two deficient brands of asparagine were supplemented

*The author is indebted to Dr. H. Bastrom and Dr. R. E. Davis of the Animal Husbandry Division for making the spectroscopic examination.

TABLE 2—The Effect on the Dry Weight Produced by Cultures[a] of Strains of Human Tubercle Bacilli by Supplementing the BAI Synthetic Medium Made from Deficient Brands of Asparagine with Traces of Mineral Elements (in the Form of Pure Compounds in Solution or as Supplied in Tap Water[b])

Brand of asparagine	Strain	Supplements	Dry weight (Gm.) produced by a single culture
Deficient #1[c]	Pn	Elements added in solution[d]	2.08
		Elements added in solution	2.08
		None[e]	0.60
		None	0.74
	C	Elements added in solution	2.01
		Elements added in solution	1.95
		None	0.55
		None	0.53
	Dt	Elements added in solution	2.03
		Elements added in solution	1.91
		None	0.61
		None	0.58
Deficient #2	Pn	Elements in solution and tap water	1.94
		Elements in solution and tap water	1.99
		Tap water alone	1.45
		Tap water alone	0.83
	C	Elements in solution and tap water	1.92
		Elements in solution and tap water	1.83
		Tap water alone	1.52
		Tap water alone	0.92
	Dt	Elements in solution and tap water	1.96
		Elements in solution and tap water	1.83
		Tap water alone	0.39
		Tap water alone	0.46
Control	Pn	Elements in solution	2.04
		Elements in solution	1.82
		None	1.62
		None	1.49
	C	Elements in solution	1.90
		Elements in solution	1.80
		None	1.62
		None	1.49
	Dt	Elements in solution	1.87
		Elements in solution	1.81
		None	1.80
		None	1.69

[a]All cultures five and one-half weeks old at time of sterilization and examination.
[b]Tap water in the proportion of 1 : 10 of total volume added to medium during its preparation.
[c]Deficient = deficient in ability to afford normal growth.
[d]Solution = solution added to medium during its preparation.
[e]Cultures grown on unsupplemented batches usually sunken at end of two to three weeks.

by the addition of the substances given in table 1 and in the amounts shown. The growth afforded by these large batches used in regular tuberculin production has been similar in all respects to that afforded by batches made of adequate brands of asparagine.

The level of the supplements required has been investigated in a very preliminary way, but there is an indication that one tenth of the level used is inadequate. Whether it is necessary to include all of the elements given in table 1 in the supplements is not known. Work intended to throw light on these problems is projected.

SUMMARY

Batches of the Bureau of Animal Industry synthetic medium made of certain brands of commercial asparagine failed to afford satisfactory growth of tubercle bacilli as used in tuberculin production. Absence of needed trace elements was considered as a possible explanation of the failure to obtain adequate growth. When batches made of these brands were supplemented with tap water, variable amounts of growth were afforded, indicating that tap water did not consistently furnish the materials needed. However, when batches of medium made from these brands were supplemented by the addition of trace quantities of various mineral elements, growth similar in all respects to that afforded by batches made of satisfactory brands of asparagine was obtained. The elements added were aluminum, barium, boron, calcium, cobalt, copper, lead, manganese, molybdenum, nickel, silver, sodium, strontium, zinc, and cobalt.

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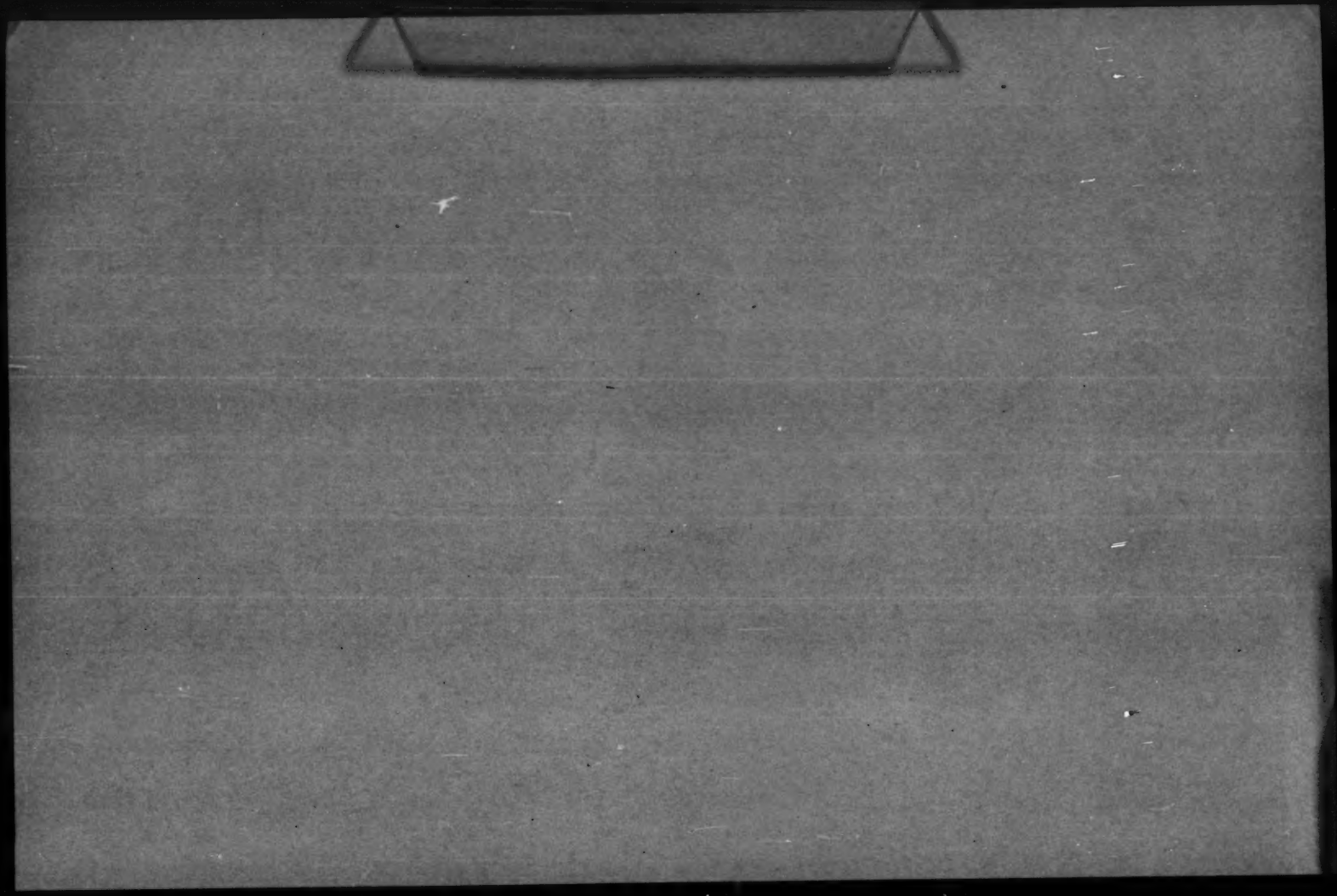
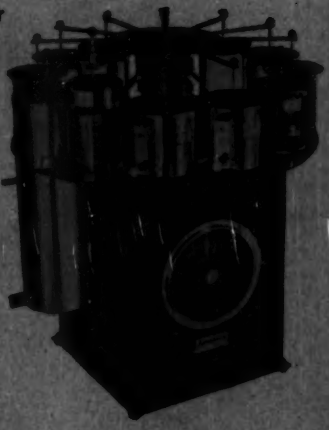


days become *hours*

Think back to the time when you were completely dependent on manual methods for histologic processing. Tissue preparation that frequently took days to do then is now done in a matter of hours. Everybody's time is saved . . . technician's, pathologist's, surgeon's, patient's, and hospital's. Tissues come through faster and better — diagnosis is prompter. The working day becomes more productive.

This is why thousands of hospitals all over the world depend, with justified confidence, on their Autotechnicons for unfailing, day-in, day-out service.



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